INTRASPECIFIC RELATIONSHIPS AMONG THE STYGOBITIC SHRIMP, *Typhlatya mitchelli*, BY ANALYZING SEQUENCE DATA FROM MITOCHONDRIAL DNA

A Thesis

by

MICHAEL SCOTT WEBB

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2003

Major Subject: Wildlife and Fisheries Sciences

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Approved as to style and content by:

Thomas Iliffe (Co-Chair of Committee)	Jaime Alvarado-Bremer (Co-Chair of Committee)
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ABSTRACT

Intraspecific Relationships among the Stygobitic Shrimp,

Typhlatya mitchelli, by Analyzing Sequence Data from Mitochondrial DNA.

(May 2003)

Michael Scott Webb, B.A., University of North Texas

Co-Chairs of Advisory Committee: Dr. Thomas Iliffe
Dr. Jaime Alvarado-Bremer

Intraspecific relationships among the anchialine cave shrimp, *Typhlatya mitchelli*, were examined by sequencing a total of 1505 bp from portions of three mitochondrial DNA genes. Cytochrome *b*, cytochrome oxidase I, and 16S rRNA were partially sequenced and analyzed for specimens from six different cenotes (water-filled caves) across the Yucatan Peninsula, Mexico. The conspecific *Typhlatya pearsei* that is sympatric with *T. mitchelli* was also sequenced and used as the outgroup. Comparisons among specimens of *T. mitchelli* yielded low sequence divergence values (0-1.7%), with the majority being less than 0.4%. Phylogenetic tree topologies reconstructed with neighbor-joining, maximum likelihood, and maximum parsimony were in agreement with respect to the resolution of deep branches. Also, there was no obvious geographic differentiation among the majority of *T. mitchelli* samples, with the exception of specimens from Cenote San Antonio Chiich (Yokdzonot, Yucatan) which all clustered into an extremely well supported monophyletic group. The level of differentiation of this group, together with the nearly total absence of differentiation among *T. mitchelli*

from distant cave systems, suggests that this is an Evolutionary Significant Unit (ESU), which may correspond to a new species. This unidentified *Typhlatya* was helpful in establishing a period in which the epigean ancestor colonized the cenotes. Based on pairwise distance data and previously published shrimp molecular clocks (Baldwin et al., 1998), *T. mitchelli* and the putative new *Typhlatya* species last shared a common ancestor between 3-5 million years ago (mid-Pliocene), while *T. mitchelli* and *T. pearsei* was approximately 7-10 mya (middle to late Miocene). The ancestor to *T. mitchelli* and the unidentified *Typhlatya* species abandoned its shallow coastal water existence in the early Pliocene and eventually expanded its range across the peninsula. Approximately 4 mya, Cenote San Antonio Chiich became isolated from the remaining gene pool thereby halting gene flow. As the regional water table fluctuated in response to the rise and fall of Pleistocene sea levels, *T. mitchelli* actively colonized the peninsula. The discovery of a single, continuous subterranean freshwater system provides for a better understanding of anchialine biogeography within the Yucatan Peninsula.

DEDICATION

I dedicate this work first and foremost to my wife, Genie. I am indebted to my wife for bringing direction and purpose to my life. She has been an unending source of understanding, love and support in addition to being my greatest friend. I thank my mother, Linda, and my father, Paul, who were the source of great support, guidance, and love. Additionally I would like to thank my stepparents, Marvel and Floyd, who also have contributed a substantial amount of time into helping me develop into the person who I am today. This work would not have been possible without these individuals and other family members and friends who have remained supportive throughout this endeavor.

ACKNOWLEDGMENTS

First, I would like to thank God, who helped me and gave me the courage to complete this scientific achievement.

I would like to thank the members of my advisory committee for their help, guidance and support. They include Drs. Thomas Iliffe, Jaime Alvarado-Bremer, Mary Wicksten and Leonard DiMichele. Special thanks goes to my mentor, Dr. Iliffe, who made this work possible by introducing me to "the world's most dangerous science." Special appreciation goes to Dr. Alvarado-Bremer for sharing his knowledge of population genetics and his expertise in the laboratory. He was indispensable in the creation and development of this work. Dr. Wicksten provided valuable help with her vast knowledge of caridean shrimps. Drs. Wicksten, DiMichele , and Jordi Vinas are very much appreciated for their critique and assistance with the writing of this manuscript. Funding for this work was provided by Dr. Iliffe's mini-grant from Texas A&M University. Support for travel abroad was made available from a Mooney Travel Grant and a Texas A&M at Galveston Graduate Student Travel Grant.

Last, but not least, I am indebted to my parents whose encouragement was unwavering and to my wife, Genie, who was a constant source of inspiration, support and love. Thank you all.

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INTRODUCTION

Our knowledge of the stygobitic (aquatic, cave-limited) fauna from the Yucatan Peninsula, Mexico is still in an infant stage. Initial investigations of the cave fauna of the Yucatan Peninsula were carried out in the 1930's by expeditions from the Carnegie Institution of Washington (Pearse et al. 1936, Pearse 1938). It was not until the 1970's that a more systematic study of the Yucatan caves occurred (Reddell, 1977). The number of troglobites (cave-limited fauna) yielded from these previous investigations totaled 34, of which only 11 were stygobites. To date, the anchialine fauna of the Yucatan Peninsula consists of 37 stygobitic species including 35 crustaceans and two fish (Iliffe, 2001).

Relatively little data has been published on studies involving anchialine taxa that are not strictly based on morphological characteristics. Fundamental issues such as systematics and population dynamics can be derived from modern molecular genetic techniques like RAPD (Randomly Amplified Polymorphic DNA), allozyme assays and DNA sequencing of nuclear and mitochondrial genes (Moritz and Hillis, 1996). The purpose of this study was to determine the intraspecific population structure for the atyid shrimp *Typhlatya mitchelli* found in the anchialine caves of Quintana Roo and Yucatan, Mexico. Information gained from this study provides a framework for the colonization and biogeographical dispersal of anchialine fauna, in particular with that of the Yucatan Peninsula.

This thesis follows the style and format of Molecular Phylogenetics and Evolution.

Technological advances over the past several decades have made scuba diving a relatively routine procedure. Cave diving is one of the more technical types of scuba diving. Improvements such as rebreathers, diver propulsion vehicles and long-lasting, high-output diving lights are enabling cave divers to reach limits that were unthinkable only a decade ago. Scientists are diving into pristine, uncharted caves and making remarkable discoveries at a rapid rate. For example, Dr. Thomas Iliffe of Texas A&M University in Galveston has discovered over 250 new taxa from caves worldwide during his career. Many of these new fauna discovered represent higher taxonomic groups. On average, one out of every four organisms described is at the genus level while one out of every ten is at the family level or higher (Iliffe, pers. comm.).

Holsinger (2000) suggested three evolutionary pathways for the development of limnostygobites, or freshwater stygobites. First, they could be derived from a marine epigean (surface-limited) ancestor being stranded by marine regression; accordingly, anchialine caves provided an "important transitional zone during marine regressions." Second, invasion of subterranean groundwaters could occur via active upstream migration into caves from spring resurgences. Finally, evolution could happen from an epigean freshwater ancestor who colonized the subterranean environment through a variety of ways, such as passive introduction into siphon-fed subterranean waters, which is widely believed to be an important route of invasion by pre-adapted organisms (Holsinger, 1988).

The anchialine caves that are of interest to this research project are located in the Yucatan Peninsula. Anchialine caves contain bodies of haline waters, usually with a

restricted exposure to open air, always with more or less extensive subterranean connections to the sea and showing noticeable marine as well as terrestrial influences (Stock et al., 1986). Cave explorer Andres Matthes estimates that there are more than 4000 cenotes in the states of Quintana Roo and Yucatan combined. Matthes also believes less than 8% of these caves have been explored; even less have undergone scientific analysis. With such a minute amount of caves actually explored and the inflated rate of biotic discovery, one can begin to realize how faunistically diverse anchialine caves are and why it is important to protect these fragile ecosystems and document the unique biota that dwell in these habitats.

The location of the Yucatan Peninsula along the Gulf Stream, between the faunally diverse anchialine caves of Central America, the Caribbean, Bahamas and Bermuda, should provide important biogeographical data pertaining to the origin and dispersal of cavernicolous fauna. This area is a flat limestone plain with no surface streams or rivers. All drainage is subterranean through an extensive network of submerged cave systems (Reddell, 1977). The lifting of the Northern Coastal Plain during the Tertiary and the higher rainfall would have allowed solution of the limestone to occur more rapidly and create the caves that are present today. These caves ultimately filled with water during the late Pleistocene era when the sea level began to rise due to the melting of glaciers. The caves of the Yucatan Peninsula contain fresh, brackish, and salt waters. Anchialine cave faunas frequently are endemic to a single cave or cave system. These truly exceptional animals have adapted to survive under unique conditions, since there is absolutely no light and very little food available. The

environment may appear harsh, but it can remain relatively stable over long periods of time. The stability of this subterranean environment provides an ideal situation for its inhabitants as they are protected against many of the perturbations that commonly affect surface environments (Holsinger, 1988). Developmentally, cave fauna lack an easily dispersed larval stage, have a low reproductive potential and have a long period of brood care (Stock, 1986). Morphologically, most anchialine taxa exhibit typical troglomorphic adaptations, which consist of varying degrees of eye loss and depigmentation, increased specialization of sensory organs, elongation of appendages and larger but fewer eggs. To date, there are nine stygobitic shrimp species that have been identified in the Yucatan Peninsula region and most have marine origins. The main objective of this research was to determine the intraspecific relationship among populations of geographically isolated atyid shrimp *Typhlatya mitchelli*, by sequencing fragments of various genes from mitochondrial DNA.

Members of family Atyidae are freshwater, but some can tolerate salinities equivalent to that of seawater. Their range is mostly tropical; however, some are found in temperate regions. Bouvier (1925) believed that atyids were probably related to bathyal marine shrimps of the family Oplophoridae. Atyidae members share morphological features, such as cheliped armature, that enable them to survive and thrive in the freshwater streams in which they inhabit. In describing *Atya innocous*, Felgenhauer and Abele (1983) note the specialized use of chemo- and mechanoreceptors in the chelipeds of the shrimp to detect trace amounts of particulate matter in the water column and then relocate to the areas in which organic debris was at the highest

concentrations. The ability to detect particulate load, current velocity and quality of food source in a poor food environment such as caves, has helped *Typhlatya* members to colonize and spread in numerous cave systems across the Western hemisphere.

The genus *Typhlatya*, having a disjunct range (see Fig. 1), has been commonly used for the development of biogeographical models and theories. Rosen (1976) postulated a vicariance model for Caribbean biogeography based on plate fragmentation. Stock's regression model (Stock, 1986) was based on a vicariant process other than plate fragmentation. Stock hypothesized the 'stranding' of marine organisms by geotectonic uplifting, followed by a gradual adaptation to mixohaline coastal groundwaters by the meiofaunal elements. Iliffe et al. (1983) suggested an origin for the genus on submerged and emergent seamounts associated with the Mid-Atlantic Ridge. Sans and Platvoet (1995) had an alternative approach based on vicariance principles that involves further evolution of each species after the disruption of the ancestral range. Of the thirteen species in the genus, four (from the Galapagos Islands, Bermuda, Ascension Island and the Caicos Islands) live in brackish or marine waters, while the remainder inhabit freshwater. Absence of clear morphological patterns within the recent species may be due to the early timing of isolation between and within lineages.

The first reported stygobitic shrimp of the Yucatan Peninsula was *Typhlatya pearsei* (Creaser, 1936). Working primarily with specimens collected by J. Reddell and associates, Hobbs and Hobbs (1976) described two new species, *Typhlatya campecheae* and *Typhlatya mitchelli,* from the Yucatan region. The current study focuses on *T. mitchelli*. Individuals of this species are translucent, white or pigmented shrimp.

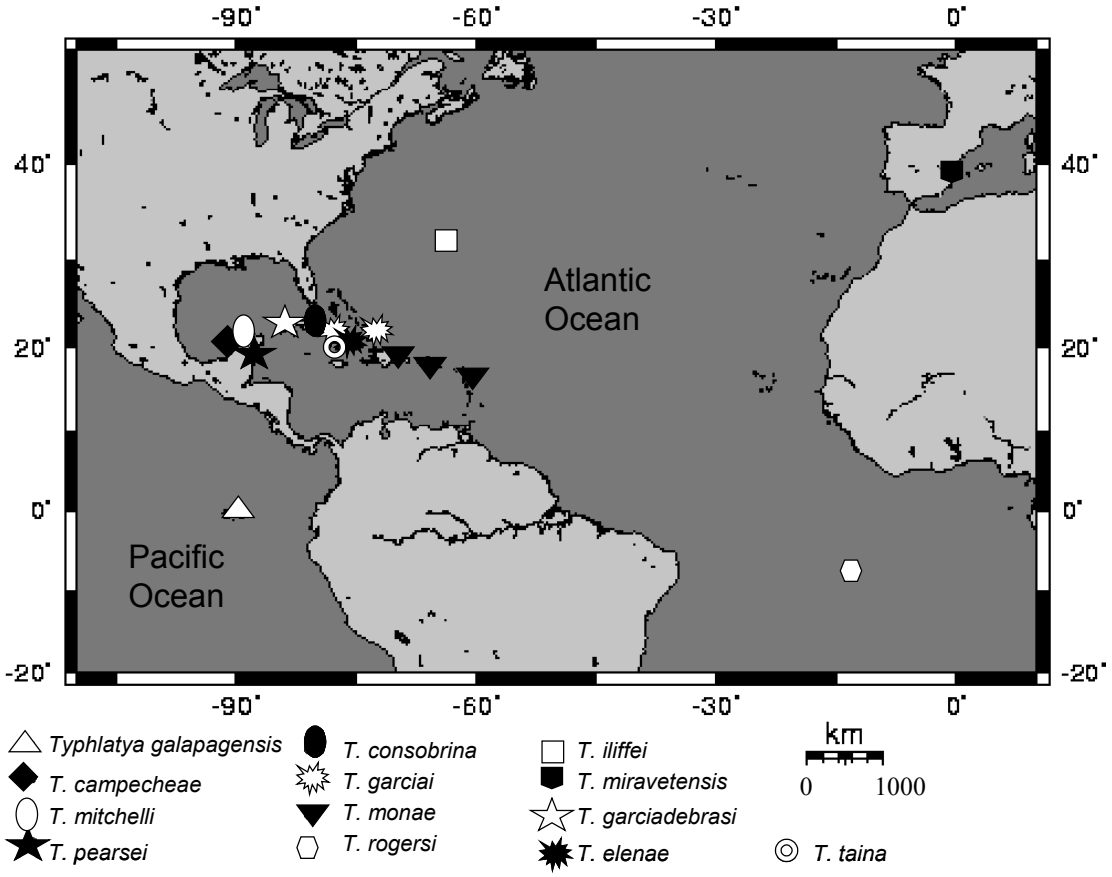

FIG. 1. Genus range for *Typhlatya*.

Pigment, which occurs only in individuals from certain localities (see Hobbs, 1979 for specific locations), is grayish to black or brown. Pigment typically occurs in individuals from localities in which the entrance pools contain dense tree roots that have become submerged. The roots appear to offer shelter and probably a food source, as shrimp have been collected with red-orange pigment that matches the color of the roots (personal observation at Cenote San Juan). Sometimes a pattern of "dark chromatophore-like specks" is prominent along the ventral margin of the carapace, the rostrum, the eye-remnant, the first abdominal tergum, and the antennular peduncle (Hobbs, 1979). The

eyes lack facets. The rostrum does not extend anteriorly beyond the eyes (Fig. 2). Their overall length is about 16 mm. The carapace length of adult females ranges from 3.4 - 4.8 mm (Hobbs and Hobbs, 1977). Presently, all taxonomic classification within this genus is determined by morphological studies.

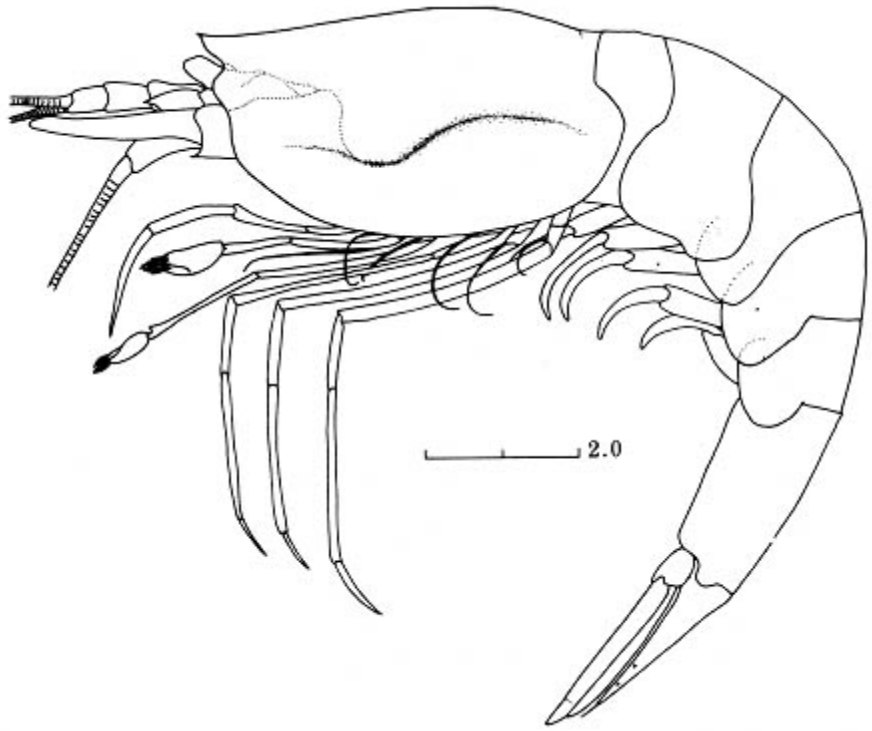

FIG. 2. Original drawing of *Typhlatya mitchelli* from Hobbs and Hobbs, 1977. Scale is in mm. Antennae and antennules are cut-off in this diagram by the artist; they are much longer in reality.

Although little is known about the life history of *T. mitchelli*, it is known to inhabit numerous cenotes in the Coastal Plain from the states of Quintana Roo and Yucatan, Mexico. The type locality for this species is Cenote Kabahchen, Mani, Yucatan, Mexico. *Typhlatya mitchelli* is intermediate, both in morphology and in

geographic position, between the Caribbean species with a short rostrum (*Typhlatya garciai* from Cuba and *Typhlatya monae* from Mona, Puerto Rico) and the single Pacific Ocean representative from the Galapagos, *Typhlatya galapagensis* (Hobbs & Hobbs, 1976). The habitat of *T. mitchelli* consists of freshwater cave pools and the subterranean networks that connect them. According to Hobbs (1979), shrimp live in situations varying from very small shallow pools to extensive underground lakes. Generally, the pools are in total darkness, but *T. mitchelli* occurs in entrance areas that receive direct light from the surface (personal observation at Cenote San Juan). Substrates of the pools consist of guano, silt, organic debris and rocks. Shrimp are found on the substrate, climbing on submerged walls along the edges of lakes or among the dense, branched root systems hanging down from the roof of cenotes, and free-swimming (Hobbs, 1979). *Typhlatya mitchelli* are often found in the same cenotes as *Typhlatya pearsei*. Both *T. pearsei* and *T. mitchelli* show identical degrees of eye reduction suggesting that they started their cavernicolous evolution at the same time (Wilkens, 1982). Due to this similarity and also that *T. pearsei* is sympatric with *T. mitchelli*, *T. pearsei* was chosen as the outgroup.

Hierarchical data analyses were employed to test the following hypotheses about relationships among the shrimp. 1) Specimens collected within the same state, Yucatan or Quintana Roo, are genetically similar. 2) Specimens collected across the Yucatan Peninsula are genetically similar.

These data would help estimate the timing of separation between populations, if any, and determine if the populations have arisen from one or more ancestral stocks.

Limestone deposits dating from the Eocene to the Holocene provide the karst terrain that is responsible for the development of the caves in this study. This extended period gives ample time for multiple colonization attempts for this species of shrimp, which is believed to have arisen from a shallow-water coastal ancestor.

There were multiple objectives under the overall aim of clarifying the intraspecific population structure of *T. mitchelli*. They were as follows: 1) obtain specimens from multiple underwater cave systems in Quintana Roo and Yucatan, Mexico, 2) assess genetic variability within and among these sites, 3) use the data to test the hypotheses stated above. By investigating the evolutionary affinities and biogeographic dispersal mechanisms for stygobitic taxa, a better understanding of the origin and development of life in anchialine caves will be achieved. This should prove to be very informative since, by definition, stygobites are strictly aquatic and cave-limited and therefore unable to cross wide regions of ocean. Over the course of two summer research expeditions in 2000 and 2001, *T. mitchelli* and its sympatric sibling species, *T. pearsei*, were found and collected in numerous cenotes across the Yucatan Peninsula (Table 1 and Fig. 3). By sequencing portions of three mitochondrial DNA genes, these specimens aided in establishing a better understanding of the anchialine cave population biogeography associated with the Yucatan Peninsula of Mexico.

Numerous studies have utilized mtDNA sequencing in order to elucidate both intra- and interspecific relationships among crustaceans. Machado et al. (1993) showed remarkable nucleotide divergences for a portion of the mtDNA gene 16S rRNA, 0–2.7% when only substitutions were observed (average of 0.7%; 2.4% when additions/deletions

TABLE 1
Cenote Names, Abbreviations, Dates, and Coordinates for Specimen Collection Sites

Cenote name	Abbreviation	Date collected	GPS coordinate	
Naharon	N	07/15/00	N 20.20	W 87.49
Carwash	CW	07/14/00	N 20.27	W 87.48
Chi Juan	CJ	07/22/00	N 20.76	W 88.93
San Antonio (Cuzama)	SAC	07/12/01	N 20.68	W 89.30
San Juan	SJ	07/18/00	N 20.73	W 89.28
Kakuel	K	07/21/00	N 20.62	W 89.57
San Antonio Chiich (Yokdzonot)	SAY	07/10/01	N 20.67	W 88.73
Aayin-Aak	AA	07/24/00	N 20.78	W 86.94
27 Steps	27	07/25/00	N 20.40	W 87.33
Santa Maria	SM	07/11/01	N 20.85	W 90.23

were included), within a single population of *Penaeus notialis*. They believe that this fragment of the large-subunit ribosomal RNA gene is a good marker for speciation studies in Crustacea, as well as for population analysis. Knowlton et al. (1993) performed a phylogenetic study on transisthmian sister taxa of snapping shrimp from the Pacific and Caribbean coasts of Panama. *Alpheus cylindricus* had a mean divergence of 8.5% for a 681 bp fragment of (COI), while another species, *Alpheus cristulifrons*, displayed an unusually elevated divergence estimate of 19.2% for the same fragment. It was concluded that COI divergence levels were approximately 2.2–2.6% per million years for seven closely related shrimp pairs based on the rising of the Isthmus of Panama between 3.0 and 3.5 million years ago. Baldwin et al. (1998) studied the phylogenetic relationship among the shrimp *Penaeus* by sequencing a 558 bp fragment of COI, the same fragment used for this intraspecific study. They revealed that intraspecific nucleotide divergences ranged between 0–3%, which allowed them to estimate a

molecular clock rate of 3% divergence per million years (MY) for COI which is slightly faster than ~2.5%/MY calculated by the Knowlton group. Additional crustaceans, other than shrimp, such as lobsters, king crabs, and amphipods have also shown high intraspecific nucleotide divergence levels for at least one of the mitochondrial genes involved in the present study (Cunningham et al., 1992; Meyran et al., 1997; Sarver et al., 1998).

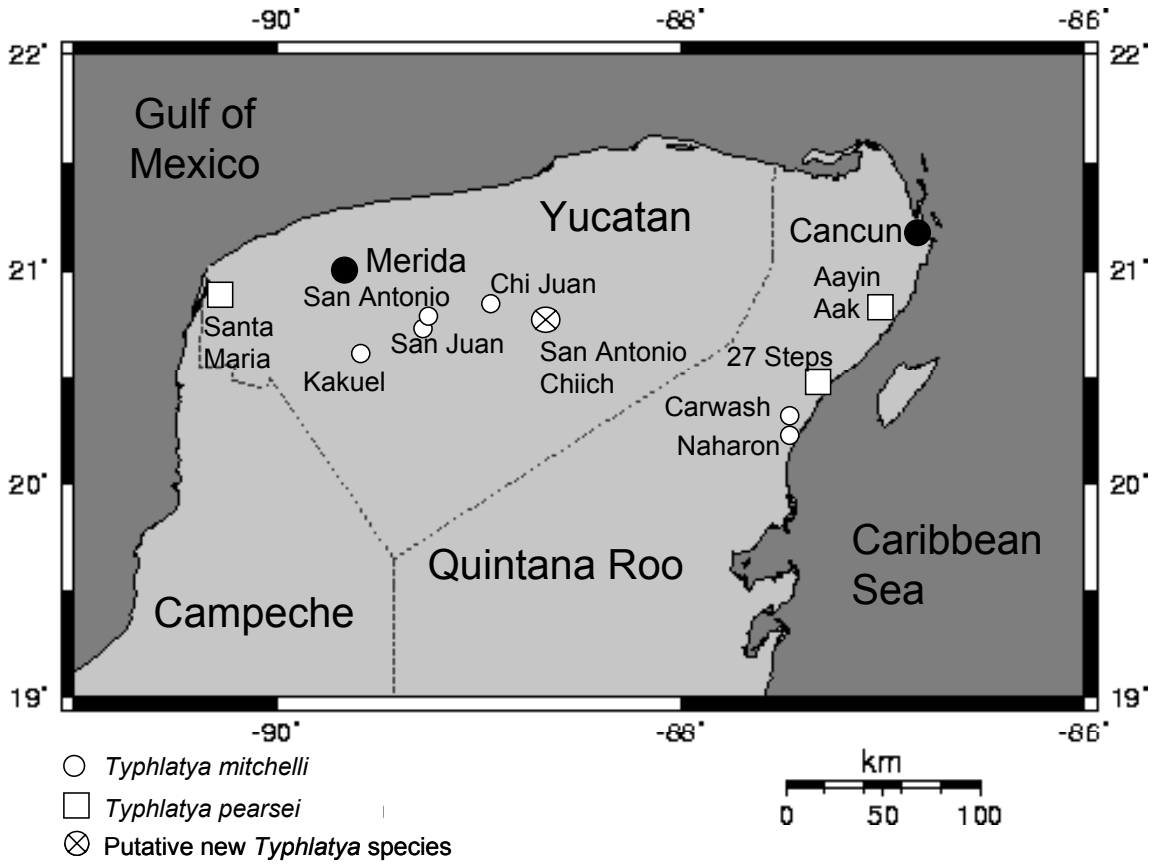

FIG. 3. Map of a portion of the Yucatan Peninsula showing the geographic localities in which shrimp were collected for this study. Details of geographic location, collections dates, and acronyms used throughout this study are given in Table 1.

MATERIALS AND METHODS

Field Work

All scientific dives conducted during the course of this research followed rules and regulations set forth by the National Speleological Society – Cave Diving Section and the American Academy of Underwater Sciences (see Appendix A for more detailed information). Dr. Thomas Iliffe, an NSS-CDS instructor was the primary dive buddy on all dives. Shrimp specimens were collected with plankton nets or 25 X 55 mm transparent vials at various locations in the cave ranging from the cavern zone to well over 500 m penetrations into the cave. Also, individuals at a few locations were collected using snorkeling gear in the entrance pool. All specimens were preserved in the field using 95% ethanol. To eliminate numerous collection tubes in the field, all specimens from a single location were stored together in a 50 ml conical tube. Upon preserving, vials were kept at room temperature.

Specimen Sorting

Since *Typhlatya mitchelli* and *Typhlatya pearsei* are sympatric with one another, all specimens had their rostrum length briefly examined using a dissecting microscope to identify species (*T. pearsei* rostrum clearly goes anteriorly beyond the eyes, whereas in *T. mitchelli* it does not). Individual specimens were then assigned a name that specified which cenote they were from and a number. For example, the third shrimp examined from Cenote San Juan would be designated SJ3. All specimens from SAY were extremely divergent and are considered a new ESU (see results). Accordingly, Fernando Alvarez, a cave shrimp taxonomist at UNAM in Mexico City, is conducting a

morphological assessment of the new ESU from Cenote San Antonio Chiich in Yokdzonot.

DNA Extractions

Where possible, three or more individuals from each population were used for molecular sequence analysis. Several cenotes each yielded less than three specimens of *T. mitchelli* (Table 2). Following the protocol described by Greig (2000), DNA was isolated by placing approximately 5 mg of abdominal tissue in a 1.5 ml microcentrifuge tube. Digestion of the tissue was accomplished by adding 200 µl of TENS solution (50 mM Tris-HCl [pH 8.0], 100 mM EDTA, 100 mM NaCl, 1% SDS) plus 20 µl of Proteinase K (10 mg/ml) and then the contents were mixed by inverting the tube. The solution was allowed to incubate for a minimum of four hours at 55°C in a heating block, periodically inverting the tube to keep it well mixed. Following incubation, 20 µl of 5 M NaCl was added and the samples were spun at 14,000 g for 10 minutes. The sample supernatants were transferred to a second microcentrifuge tube and mixed with two volumes of cold (-20°C) 100% ethanol to precipitate DNA. Spinning the samples for an additional 10 minutes at 14,000 g then pelleted the DNA. The resulting supernatant was poured off and the DNA pellets were washed with 300 µl of cold 70% ethanol and allowed to air-dry overnight. DNA was resuspended in 100 µl of TE buffer (10 mM Tris-HCl [pH 8.0], 1mM EDTA). Samples were stored at 4°C until ready for use. A 1 µl aliquot of genomic DNA was used as starting template for the 12.5 µl PCR reaction. Some reactions required dilutions up to 1:100 in order to achieve the desired result.

TABLE 2
Sequencing Sample Size for Each Cenote

Cenote name/ Abbreviation	Cytochrome *b* (483 bp)	Cytochrome Oxidase I (542 bp)	16S rRNA (480 bp)
Typhlatya mitchelli			
Naharon/ N	1	1	1
Carwash/ CW	4	3	3
Chi Juan/ CJ	6	3	3
San Antonio (Cuzama)/ SAC	2	2	2
San Juan/ SJ	7	3	3
Kakuel/ K	5	3	3
Putative new ESU			
San Antonio Chiich (Yokdzonot)/ SAY	5	0	5
Typhlatya pearsei			
Aayin Aak/ AA	1	1	1
27 Steps/ 27	1	1	1
Santa Maria/ SM	1	1	1
Total	33	18	23

PCR Reactions

PCR primers (Table 3) for cytochrome *b* (Cyb), 16S rRNA (16S), and cytochrome oxidase I (CO) for mitochondrial DNA (mtDNA) were chosen based on their success rate among other crustacean studies (Simon et al., 1991; Baldwin et al., 1998; Harrison, 2001). PCR reactions were conducted in 12.5 µl volumes containing: 6.9 µl dH_2O, 1.25 µl 10X buffer, 2 µl MgCl (final concentration of 4 mM), 0.25 µl 10 mM dNTPs, 0.5 µl of each primer (10 pM/µl), 1 µl template, and 0.1 µl (0.5 U/sample) of AmpliTaq® polymerase (Roche, Branchburg, NJ). The PCR reactions were performed in an Eppendorf mastercycler personal (Eppendorf Scientific, Inc., Westbury, NY) with the following parameters for the mitochondrial gene cytochrome *b*: an initial denaturing step at 94°C for 2 minutes, followed by 35 cycles of the following profile:

denaturing step for 20 seconds at 94°C, annealing step for 30 seconds at 50°C, and an extension step for 30 seconds (+ 1 sec/cycle) at 72°C. The final extension phase was for 3 minutes at 72°C. For cytochrome oxidase I (COI) and 16S rRNA, the following parameters were used: an initial denaturing step at 94°C for 3 minutes, followed by 35 cycles of the following profile: denaturing for 45 seconds at 94°C, annealing for 45 seconds at 47°C, and an extension step for 60 seconds (+ 1 sec/cycle) at 72°C. The final extension phase was for 5 minutes at 72°C. All PCR reactions were run concurrently with a negative control (template-free reaction) to assure no contamination had occurred.

TABLE 3
Primer Names, Source, and Nucleotide Sequence

Primer	Reference	Sequence
Cyb1	Harrison, 2001	5’ ATT TGT CGA GAT GTR AAY TAY GG 3’
Cyb2	Harrison, 2001	5’ AAA TAT CAT TCN GGY TGR ATR TG 3’
16Sar	Simon et al., 1991	5’ CGC CTG TTT ATC AAA AAC AT 3’
16Sbr	Simon et al., 1991	5’ CCG GTC TGA ACT CAG ATC ACG T 3’
CO9	Baldwin et al., 1998	5’ TTC GGT CAY CCA GAA GTM TAT 3’
CO10	Baldwin et al., 1998	5’ TAA GCG TCT GGG TAG TCT GAR TAK CG 3’

All products were run on a 1.5% agarose gels (Type I, Sigma-Aldrich, St. Louis, MO) for 30 minutes at 100 V to verify quality of fragment amplifications and visualized with ethidium bromide using ultraviolet light. Photographs of gels were taken with a Kodak DC 120 Zoom digital camera and images were analyzed with Kodak Digital Science™ 1D image analysis software version 3.0.2 (Eastman Kodak Company, Rochester, NY). Combinations of various universal primers for the mitochondrial gene

12S rRNA and nuclear elongation factor and beta tubulin genes proved unsuccessful in multiple attempts of gene amplification.

Purification of PCR Products

All successful PCR products were cleansed with ExoSAP-IT (USB, Cleveland, OH). Following the manufacturer recommendations, 5 µl of PCR product were mixed with 2 µl of enzyme and placed in a thermal cycler for 15 minutes at 37°C followed by an inactivation step for 30 minutes at 80°C. ExoSAP-IT consists of two hydrolytic enzymes, exonuclease I which degrades any single-stranded DNA and shrimp alkaline phosphatase which hydrolyzes any remaining dNTPs.

Cycle Sequence Reactions

Two microliters of purified PCR products were added to 0.2 ml tubes containing 4 µl of Big Dye™ Terminator Ready Reaction Mix (Applied Biosystems, Foster City, CA) and 4 µl of diluted 1X primer (1:3). Fluorescent-labeled dideoxynucleotide triphosphates were used for cycle sequencing. Reaction parameters were 25 cycles of denaturing for 10 seconds at 96°C, followed by annealing for 5 seconds at 50°C and extension for 4 minutes at 60°C. Unincorporated nucleotides were removed from cycle sequencing products using the RapXtract II kit by Prolinx (Bothell, WA). Briefly, the 10 µl cycle sequencing products were added to individual wells on a 96-well plate containing paramagnetic particles and vortexed for 45 seconds. Samples were retrieved and diluted with 20 µl dH_2O before being loaded into the sequencing tray. Capillary electrophoresis was conducted using modified settings associated with POP6 (Applied Biosystems, Foster City, CA), according to recommendations in RapXtract II kit. The

samples were injected into the capillary using 3.5 kV for 40 seconds. An ABI Prism™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) was used to collect sequence data.

Data Analysis

Visual inspection of chromatographs were performed on all sequences to validate nucleotide designations and to clarify any ambiguous sites called by the Sequencing Analysis software associated with the ABI Prism™ 310 Genetic Analyzer. To determine if any mutations were introduced through PCR reactions, reverse complement strands were run on randomly selected sequences. Sequences were then edited in BioEdit (Hall, 1999). Multiple sequence alignments were performed with ClustalW (Thompson et al., 1994). All sequences were then submitted to the GenBank (see Table 4 for accession numbers).

Statistics on nucleotide composition, interspecific, and intraspecific pairwise comparisons were computed with MEGA (Kumar et al., 1993). Pairwise distances (d) were obtained among all individuals, while pairwise distances corrected within groups (Da) were found among all subpopulations. Haplotype frequency and diversity were sought using the program DnaSP, version 3.51 (Rozas and Rozas, 1999). Neighbor-joining, maximum likelihood, and maximum parsimony methods were employed in the search to find an optimal phylogenetic tree with the software programs MEGA, PHYLIP (Felsenstein, 1993), and PAUP (Swofford, 1998). The robustness of tree topologies was tested with the bootstrapping method (Felsenstein, 1985), which involves creating a new data set by resampling a certain number of characters in the original data randomly and

TABLE 4
GenBank Accession Numbers to All Specimens

Organism	Gene	GenBank Accession Numbers
Typhlatya mitchelli	Cyt *b*	AF512032 – AF512056
	COI	AF513509 – AF513523
	16S rRNA	AF513524 – AF513538
Typhlatya pearsei	Cyt *b*	AY115531 – AY115533
	COI	AY115534 – AY115536
	16S rRNA	AY115537 – AY115539
Typhlatya sp.	Cyt *b*	AY115540 – AY115544
	16S rRNA	AY115545 – AY115549

leaving some characters out while duplicating others. The random variation of the results from analyzing these bootstrapped data sets can be shown statistically to be typical of the variation that one would obtain from collecting new data sets. Neighbor-joining trees were created with MEGA, version 2.1 using the Tamura-Nei nucleotide evolutionary model (1993), using the pairwise deletion option and 500 bootstrap iterations. The Tamura-Nei model (1993) was chosen as it accounts for differences between nucleotides and inequality of their frequencies. It also distinguishes between transition and transversion frequencies, but assumes substitution rates are equal at all sites. Neighbor-joining (Saitou and Nei, 1987) does not assume molecular rate constancy and the topology showing the smallest value of the sum of all branches is chosen as an estimate of the correct tree. Maximum likelihood trees were created with the program DNAML in PHYLIP, version 3.57 using the default parameters and 100 bootstrap iterations. Maximum likelihood (Felsenstein, 1981) uses a robust algorithm that tends to outperform other tree-building methods such as parsimony and additive

distance (Moritz and Hillis, 1996). This method incorporates the following assumptions: 1) Each site in the sequence evolves independently. 2) Lineages evolve at different rates. 3) Each site undergoes an expected rate of substitution. 4) All relevant sites are included in the analysis, not just phylogenetically informative sites. 5) Transitions and transversions occur at different rates. Maximum parsimony trees were created using PAUP, version 4.0b 10. A heuristic search was employed with 500 bootstrap iterations in an effort to find the most parsimonious tree. Starting trees were retrieved by random stepwise addition. Maximum parsimony is a character-based method that infers the topology that requires the smallest number of evolutionary changes (e.g., nucleotide substitutions). It follows Ockham's razor in that the best hypothesis is the one requiring the smallest number of assumptions.

The use of these gene phylogenies of mtDNA together with geographic information on the populations sampled provides a means for evaluating the genetic structure of populations and will provide a better understanding of the anchialine cave population biogeography associated with the Yucatan Peninsula of Mexico.

RESULTS

Sequence Orthology of Mitochondrial Genes

Representative sequences for each gene were submitted to GenBank (using blastn) in order to find orthologous sequences. The highest matches for all three were with counterpart genes in other invertebrates (including Penaeid shrimps). An analysis of the pattern of nucleotide substitutions among haplotypes showed that these were biased toward transitions in all three mtDNA genes surveyed (R=4.6 when all data was combined). Furthermore, similar to other shrimp mitochondrial DNA sequences (Baldwin et al., 1998; Shank et al., 1999), the A/T content is noticeably high at 66% for cytochrome *b*, 64.1% for cytochrome oxidase I, and 68.6% for 16S rRNA. Finally, the positional biases established for these genes are similar to those found in other surveys of animal mtDNA (Yoder et al., 1996). Overall, these patterns of base composition (high adenine/thymine content and bias against guanine/cytosine) and base substitution bias provide assurances that these DNA sequences are not nuclear pseudogenes (Zhang and Hewitt, 1996).

Cytochrome b *Variation in* Typhlatya mitchelli

Of the three-mitochondrial DNA genes sequenced to characterize variation in *Typhlatya*, the 483 base pair segment of the cytochrome *b* gene was the most variable. A total of 11 variable sites, corresponding to ten transitions and one transversion, were found among the samples of *T. mitchelli* (see Appendix E for complete sequences). Eight of the 11 sites occurred at the third codon position, while two were at the first position and one at the second position. There was a slight bias against guanine in the

second position and an overwhelming bias against it in the third position (see Table 5). Thymine was the favored nucleotide at all three positions, especially at the second position in which it was present over 47% of the time. Contributions towards the overall nucleotide composition from specimens of *T. pearsei* (SM6, AA3 and 27-1p) and the unidentified *Typhlatya* species (specimens from Cenote San Antonio Chiich in Yokdzonot or SAY) are also included in Table 5. The 11 variable nucleotide sites caused four amino acid substitutions (A>V, A>T, L>F, and M>V).

TABLE 5
Nucleotide Percentages for Each Codon Position of the Cytochrome *b* Sequences (L-strand)

Nucleotide Composition for cytochrome *b* gene				
	Typhlatya mitchelli only (*T. pearsei* + SAY samples included)			
	T	C	A	G
position 1	31.7 (31.5)	19.9 (19.8)	26.7 (26.3)	21.7 (22.4)
position 2	47.3 (46.7)	21.7 (22.1)	17.4 (17.5)	13.7 (13.6)
position 3	40.0 (37.9)	19.9 (21.4)	35.2 (35.6)	4.9 (5.1)
average	39.6 (38.7)	20.5 (21.1)	26.4 (26.5)	13.4 (13.7)
A/T composition	66.0 (65.2)			

These polymorphic sites defined eight haplotypes among the sample (n=25) corresponding to a value of haplotypic diversity of 0.730 ± 0.063. The frequency of cytochrome *b* haplotypes is given in Table 6. Seven of the 11 polymorphic sites differentiated two specimens from Cenote Kakuel (K3, K5) from the rest of the haplotypes. In addition, six sites were parsimony-informative and five of these were unique to two distinct haplotypes corresponding to two Kakuel specimens.

TABLE 6
Haplotype Frequencies for *Typhlatya mitchelli* Using Cytochrome *b* Sequences

	1	2	3	4	5	6	7	8	Total
Subpopulation									n=25
N	1								1
CW	4								4
CJ		6							6
SAC	2								2
SJ	2	4		1					7
K			1		1	1	1	1	5

Due to the paucity of polymorphic sites, both transitions and transversions were included in all phylogenetic analyses. Tamura-Nei (1993) distance values of sequence ranged from 0 to 1.7% seen among all *T. mitchelli* specimens (Appendix B), with an overall average = 0.37 ± 0.13%. Among subpopulations of *Typhlatya mitchelli*, mean corrected divergence values (Da) ranged from 0 to 0.31% (Table 7).

Individual *Typhlatya mitchelli* shrimp differed between 13.5 and 13.8% from the SAY sample and between 24.8 and 27.1% compared to the *T. pearsei* outgroup (Appendix B). Additionally, when the pairwise comparisons were limited to transversions the nucleotide divergence values dropped to 2.8 to 3.0% between *T. mitchelli* and the *Typhlatya* specimens from SAY, and to 5.7 to 6.4% between *T. mitchelli* and *T. pearsei* specimens.

Mean corrected pairwise distances between subpopulations (Table 7) were used to clarify the relationships among subpopulations with a neighbor-joining tree (Fig. 4). Individual specimen tree topologies were all similar when obtained with neighbor-

TABLE 7

Cytochrome *b* Pairwise Distances Corrected within Groups (Da) among *Typhlatya mitchelli* Subpopulations Using the Tamura-Nei Evolutionary Model

Naharon								
Carwash	0.0000							
Chi Juan	0.0021	0.0021						
SAC	0.0000	0.0000	0.0021					
San Juan	0.0006	0.0006	0.0003	0.0006				
Kakuel	0.0015	0.0015	0.0031	0.0015	0.0018			
SAY	0.1354	0.1354	0.1378	0.1354	0.1363	0.1312		
T. pearsei	0.2426	0.2426	0.2453	0.2426	0.2428	0.2402	0.2438	
	Naharon	Carwash	Chi Juan	SAC	San Juan	Kakuel	SAY	*T. pearsei*

joining (Fig. 5), maximum likelihood and maximum parsimony. No geographic structuring was revealed among all samples of *T. mitchelli*. The only branch strongly supported among the *T. mitchelli* specimens, clustered the unique haplotypes of Cenote Kakuel. Specimen K4 was weakly supported in between the K3, K5 haplotypes and the remaining *T. mitchelli* with bootstrap scores of 71, 87 and 73% in the different trees. The Negative Natural Log Likelihood or –LnLi score was –1320.91 and 2685 trees were examined to find the maximum likelihood tree. Maximum parsimony yielded a single parsimonious tree (Length = 153; Consistency Index = 0.954; RI = 0.985; RC = 0.940). In all trees, the remainder of the Kakuel haplotypes fell onto terminal branches. Also of interest was the consistencies of the internal branching of the outgroup. The *T. pearsei* specimen SM6 from the western coast of the Yucatan and the two *T. pearsei* from the eastern coast, AA3 and 27-1p formed distinct groups.

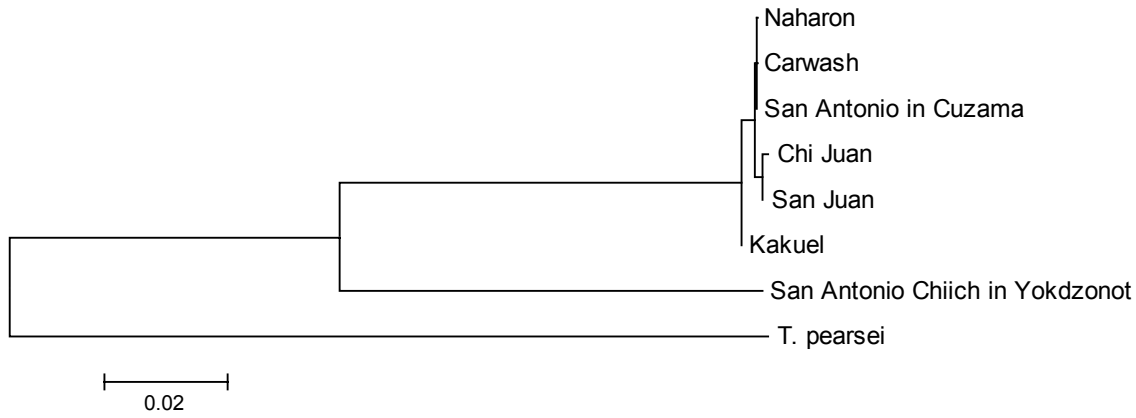

FIG. 4. Neighbor-joining tree of subpopulations created with MEGA using Da pairwise distances of cytochrome *b* sequences.

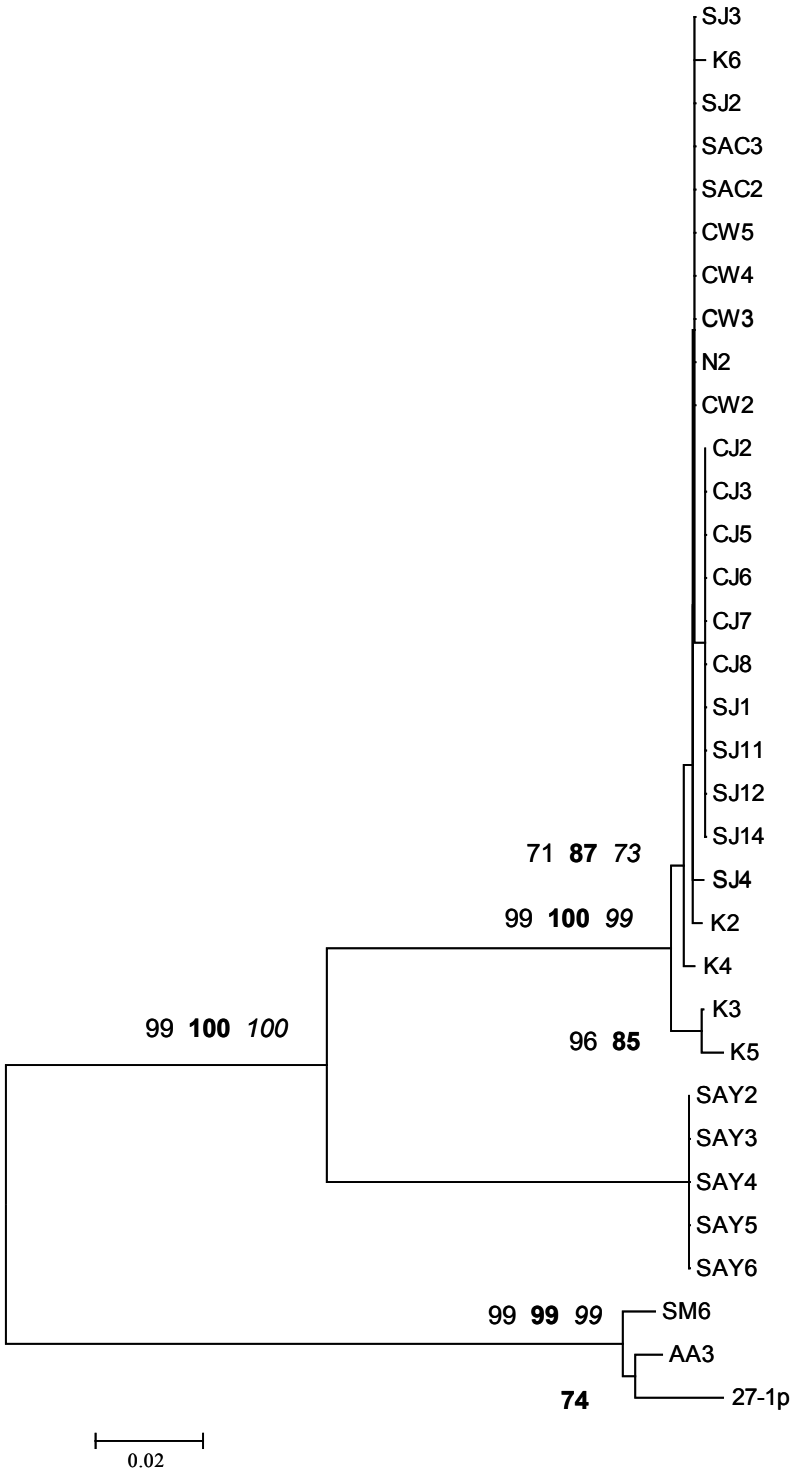

FIG. 5. Neighbor-joining tree of individual specimens developed using cytochrome *b* sequences and Tamura-Nei evolutionary model. Bootstrap scores above 70 shown. Maximum parsimony bootstrap scores are shown in bold, while maximum likelihood scores are italicized.

Cytochrome Oxidase I Variation in Typhlatya mitchelli

Similar to cytochrome *b* results, the 542 base pair section of the cytochrome oxidase I gene (COI) showed no insertions or deletions across the subpopulations or when evaluated against *Typhlatya pearsei* (for complete sequences see Appendix F). There were a total of four haplotypes revealed among the fifteen *T. mitchelli* specimens sequenced for COI. The haplotype diversity for this particular set of sequences was 0.467 ± 0.148. The frequency of haplotypes is included in Table 8. There were only seven variable sites and all were transitions. Similar to cytochrome *b*, six of the seven variable sites distinguished the unique haplotype from Cenote Kakuel (K3) from the remaining haplotypes. All of the substitutions occurred at the third codon position except for one, which occurred at the first position. There was a still a slight bias against guanine in the second position and an overwhelming bias against it in the third position (see Table 9). However, thymine was only the favored nucleotide in the last two positions for this gene fragment, while adenine was the preferred nucleotide at the

TABLE 8
Haplotype Frequencies for *Typhlatya mitchelli* Using COI Sequences

	1	2	3	4	Total
Subpopulation					n=15
N		1			1
CW	3				3
CJ	3				3
SAC	2				2
SJ	2		1		3
K	1		1	1	3

first position, followed by guanine and then thymine. The seven variable nucleotide sites caused no changes in amino acid translation.

TABLE 9
Nucleotide Percentages at All Codon Positions for COI Sequences (L-strand)

Nucleotide Composition for COI gene				
	Typhlatya mitchelli only (including *T. pearsei*)			
	T	C	A	G
position 1	24.4 (24.0)	17.3 (17.7)	31.1 (31.1)	27.2 (27.3)
position 2	42.5 (42.5)	22.1 (22.1)	19.3 (19.3)	16.0 (16.1)
position 3	41.0 (37.7)	19.8 (22.3)	33.7 (34.3)	5.5 (5.7)
average	36.0 (34.7)	19.7 (20.7)	28.1 (28.2)	16.2 (16.3)
A/T composition	64.1 (62.9)			

Since a smaller number of variable nucleotide sites existed in this gene fragment compared to cytochrome *b*, all phylogenetic analyses also used both transitions and transversions. COI pairwise comparisons for all specimens, including the outgroup, are summarized in Appendix C. Tamura-Nei sequence divergence values ranged from 0 to 1.3% among all *T. mitchelli* specimens, with the majority being identical. The overall nucleotide divergence mean was 0.21± 0.08%. The different subpopulations show lower mean corrected divergence values (Da) than that seen with cytochrome *b* sequences, 0 to 0.25% (Table 10).

Individual *Typhlatya mitchelli* specimens differed by 20.7 ± 0.3% compared to the *T. pearsei* outgroup. Unfortunately, this gene fragment could not be amplified for

TABLE 10
COI Pairwise Distances (Da) of *Typhlatya mitchelli* Subpopulations

Naharon							
Carwash	0.0019						
Chi Juan	0.0019	0.0000					
SAC	0.0019	0.0000	0.0000				
San Juan	0.0019	0.0000	0.0000	0.0000			
Kakuel	0.0025	0.0006	0.0006	0.0006	0.0002		
T. pearsei	0.1997	0.1988	0.1988	0.1988	0.1982	0.1951	
	Naharon	Carwash	Chi Juan	SAC	San Juan	Kakuel	*T. pearsei*

specimens from Cenote San Antonio Chiich in Yokdzonot (SAY) despite numerous attempts, which precluded these comparisons. Using only transversions, pairwise comparisons between *T. mitchelli* and the outgroup yielded a divergence value of 6.1%.

The neighbor-joining tree for the subpopulations given in Figure 6 was created using the distance matrix from Table 10. Tree topologies for individual haplotypes from the COI fragment (Fig. 7) were slightly different than that obtained from cytochrome *b* data. Similar to cytochrome *b*, all three tree-building methods generated similar tree topologies with similar bootstrap values. Again, similar to cytochrome *b* tree topologies in which three distinct groupings were found (K3/K5, K4, and all remaining *Typhlatya mitchelli*), there were three different groups elucidated among the individuals of *T. mitchelli*. The specimen known as K3 emerged again as the most divergent and produced strong bootstrap support in all trees (score = 100). Specimen K4, only weakly supported in the cytochrome *b* trees as unique, was now clustered with a single specimen from Cenote San Juan (SJ2) in a well supported branch (average bootstrap score = 82). All remaining *T. mitchelli* specimens, including the remaining specimen from Kakuel (K2), once again clustered among the terminal branches. Maximum likelihood

examined 680 trees and produced a –LnLi score of –1182.19. The single most parsimonious tree yielded a length of 108 steps and a consistency index of 0.991 (RI = 0.995; RC = 0.985). Similar to earlier findings, the outgroup SM6 specimen from the western coast of the Yucatan and the two *T. pearsei* from the eastern coast, AA3 and 27-1p, still formed distinct groups.

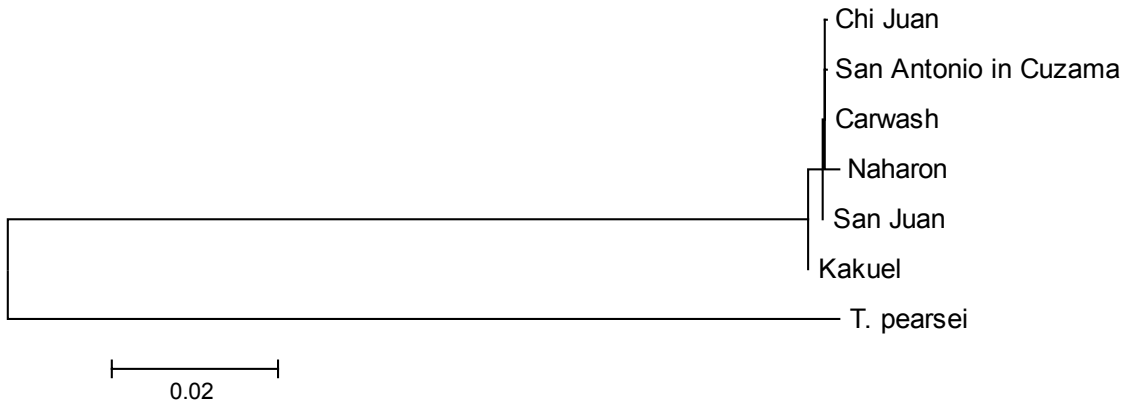

FIG. 6. Neighbor-joining tree of subpopulations created with the program MEGA using Da pairwise distances of COI sequences.

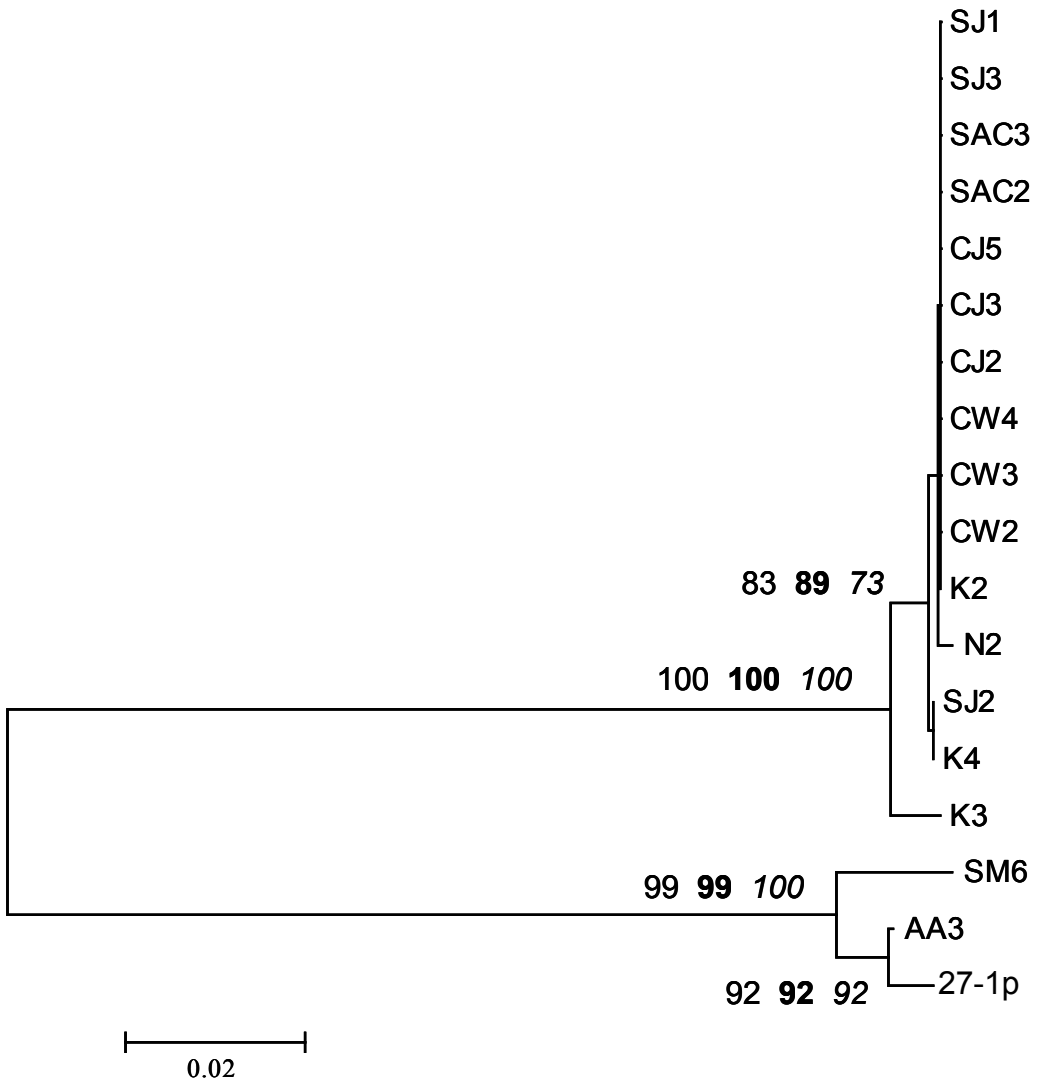

FIG. 7. Neighbor-joining tree of individual specimens using COI sequences. Tree was created in MEGA with bootstrap scores shown. Maximum parsimony bootstrap scores are shown in bold, while maximum likelihood scores are italicized.

16S rRNA Variation in Typhlatya mitchelli

The 480 base pair section of the 16S rRNA gene showed no insertions or deletions among the subpopulations of *T. mitchelli* (see Appendix G for complete sequences). However, compared to the SAY specimens and *T. pearsei*, one and four

insertions/deletions, respectively, were necessary to maintain the proper sequence alignment. Among the *T. mitchelli* sample, there were only two variable nucleotide sites, one transition and one transversion that defined three haplotypes. The transition site was a parsimony-informative site and included the two shrimp from Cenote San Antonio in Cuzama (SAC2, SAC3) and a single specimen from Cenote San Juan in Homun (SJ3). The single transversion distinguished specimen CW2. The remaining 11 of 15 *T. mitchelli* specimen's sequences belong to a ubiquitous haplotype. Overall, the haplotype diversity for this set of sequences was 0.448 ± 0.134. Matching the previous gene fragments, the A/T content was still high at 68.6% (Table 11). Adenine was the overall favored nucleotide at 37.1%, while guanine was again the least favored at 10.0%.

TABLE 11
Average Nucleotide Percentages for 16S rRNA Sequences

Nucleotide Composition for 16S rRNA gene				
	Typhlatya mitchelli only (*T. pearsei* + SAY samples included)			
	T	C	A	G
average	31.5 (30.6)	21.4 (21.7)	37.1 (37.4)	10.0 (10.4)
A/T composition	68.6 (68.0)			

Pairwise distances using both transitions and transversions among 16S rRNA for all specimens, including the outgroup, are summarized in Appendix D. Sequence divergence values among all *T. mitchelli* were very low and ranged between 0 and 0.4% between all specimens, with a mean of 0.09 ± 0.07%. The distance of *Typhlatya*

mitchelli specimens from the SAY specimens varied between 5.4 and 5.9% and between 15.6 and 16.5% compared to the *T. pearsei* specimens. Subpopulation pairwise distances were extremely low as noted in Table 12. Using only transversions (data not shown), nucleotide divergence estimates ranged from 1.5 to 1.7% between *T. mitchelli* and the *Typhlatya* from SAY, while it varied from 3.2 to 3.4% between *T. mitchelli* and *T. pearsei.*

TABLE 12
Subpopulation Pairwise Distances (Da) for *Typhlatya mitchelli* Using 16S Sequences

Naharon								
Carwash	0.0000							
Chi Juan	0.0000	0.0000						
SAC	0.0021	0.0021	0.0021					
San Juan	0.0000	0.0000	0.0000	0.0007				
Kakuel	0.0000	0.0000	0.0000	0.0021	0.0000			
SAY	0.0541	0.0541	0.0541	0.0565	0.0542	0.0541		
T. pearsei	0.1595	0.1586	0.1595	0.1564	0.1578	0.1595	0.1446	
	Naharon	Carwash	Chi Juan	SAC	San Juan	Kakuel	SAY	*T. pearsei*

The neighbor-joining tree for the subpopulations (Fig. 8) was unique compared to the previous genes trees. Tree topologies yielded for individual specimens using 16S rRNA sequences (Fig. 9) were also quite different from the previous gene fragments but did manage to match each other for this particular set of sequences. This gene fragment did not support the distinctiveness of the Kakuel lineage as seen in cytochrome *b* and cytochrome oxidase I. There were only two distinct groupings found among all *Typhlatya mitchelli* specimens, yet the unique haplotype K3 from previous trees was not a part of this unique group, but mixed in with the remaining *T. mitchelli*. The small grouping, which was well supported with a bootstrap score of 99 in the neighbor-joining

method, featured both specimens from Cenote San Antonio in Cuzama (SAC) and one from Cenote San Juan (SJ3). It must be noted that a single transition site in the sequenced portion of 16S rRNA supported this small group. The 16S rRNA maximum likelihood was chosen after 1507 trees were examined and produced a –LnLi score of –997.41. The single most parsimonious tree produced had a length of 85 steps and a CI of 0.953 (RI = 0.984; RC = 0.937). Once again, SM6 was separated from the other two *T. pearsei* specimens, AA3 and 27-1p.

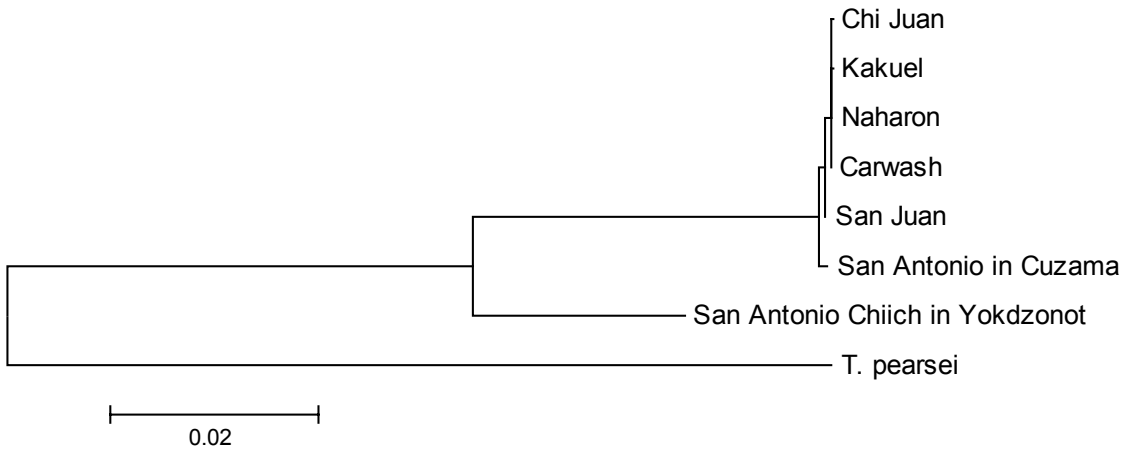

FIG. 8. Neighbor-joining tree of subpopulations developed with Da pairwise distances using 16S rRNA sequences.

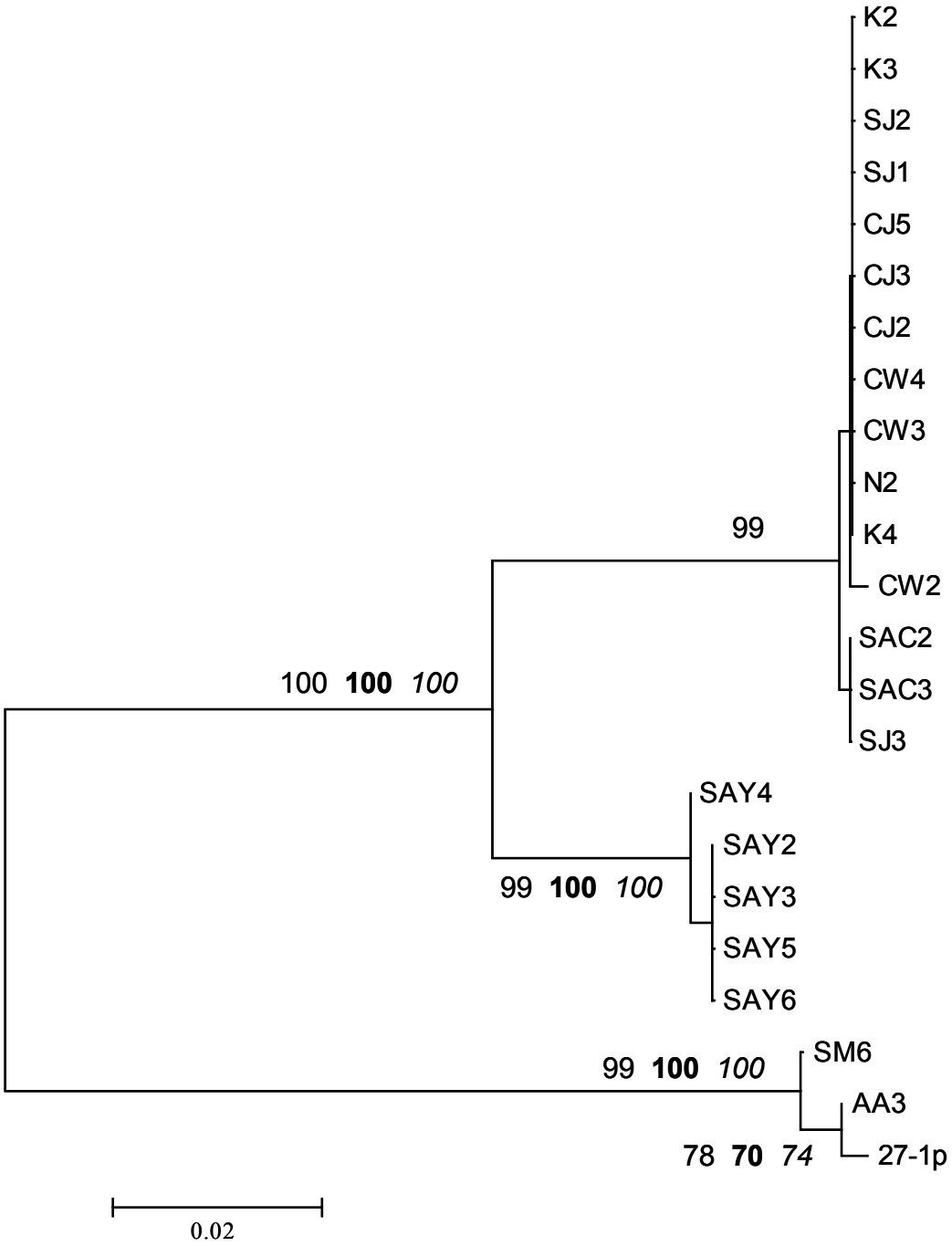

FIG. 9. Neighbor-joining tree of individual specimens using 16S rRNA sequences. The Tamura-Nei model of nucleotide evolution was used with 500 bootstrap iterations. Maximum parsimony bootstrap scores are shown in bold and maximum likelihood scores are italicized.

To better summarize the patterns of variation and differentiation among all three mitochondrial genes surveyed in this study, a single continuous sequence of 1505 bp for *T. mitchelli* and 1509 bp for *T. pearsei* was created for each of the 15 specimens of *T. mitchelli* and three *T. pearsei* that had all three gene fragments sequenced. For *T. mitchelli*, only 17 polymorphic sites were present in this segment giving an overall nucleotide diversity of 0.21 ± 0.08%. When comparing all combined sequences for the 15 *T. mitchelli* specimens, a total of 9 haplotypes were unveiled corresponding to an overall haplotype (gene) diversity of 0.905 ± 0.054 (Table 13). However, most haplotypes differed from any other haplotype by only one or two nucleotide changes. The exception lay with the distinct haplotype known as K3, in which it was unique in having or sharing 11 of the 17 polymorphic sites.

TABLE 13
Haplotype Frequencies for Combined Sequences of All 3 Gene Fragments

	1	2	3	4	5	6	7	8	9	Total
N	1									1
CW		1	2							3
CJ				3						3
SAC					2					2
SJ				1	1	1				3
K							1	1	1	3
										15

DISCUSSION

Intraspecific variation of Typhlatya mitchelli

The trees generated for the two protein-coding genes, cytochrome *b* and COI, were the most similar. Specifically, in both gene trees, individuals from Cenote Kakuel emerged as two strongly supported lineages, distinct from all remaining haplotypes of *T. mitchelli.* Due to the extremely low amount of nucleotide divergence (2 variable positions out of a total of 480 bp sequenced), the topology of the consensus tree obtained from 16S rRNA data was quite different from the consensus trees obtained from cytochrome *b* and COI data. All three gene fragments were concordant with respect to the topology for the outgroup in which the two eastern coast *T. pearsei* (AA3 and 27-1p) specimens emerged as more closely related to each other with respect to the west coast specimen SM6. The neighbor-joining trees built with the pairwise distances using corrected means within each subpopulation yielded similar topologies to the haplotype trees further supporting the overall relationship among cave systems and taxa.

One of the most relevant findings of this study was that samples of *Typhlatya mitchelli*, collected in vast geographical area of the Yucatan Peninsula had unusually low amounts of variation in all three mitochondrial regions surveyed. Polymorphic variations were highest for the cytochrome *b* fragment, followed by the COI fragment and finally 16S. No insertions or deletions (indels) were observed among *T. mitchelli* specimens when examining all gene sequences; however, four indels (all contained in 16S rRNA gene) were found when comparing *T. pearsei* to *T. mitchelli*. Given that 16S is not a protein-coding gene, indels are more likely to occur here (Machado et al., 1993).

In the pairwise comparison of a ~400 bp segment of 16S rRNA from 6 individuals, Machado et al. found 11 indels in *Penaeus notialis*. When a representative *P. notialis* sequence was aligned against three additional taxa (*Penaeus schmitti*, *Drosophila yakuba*, and *Artemia salina*), indels of 3, 18, and 34 were required to properly align these sequences.

Geographically separated samples of *Typhlatya mitchelli* collected across the Yucatan Peninsula had unusually low levels of genetic differentiation. Given that there are no surface streams or rivers in the Yucatan, the absence of genetic differentiation among *T. mitchelli* samples, suggest potential gene flow which is not congruent with the geographic distance that separates cave entrances nor the geological timing suggested for the formation of these cave systems (see below). At the surface, the cenotes appear to be geographically isolated from each other (some more than 200 km in this study). However, below the surface in the limestone platform, thousands of kilometers of intertwining cave passages exist. Information collected over the last five years indicates that one cave system, known as Sistema Ox Bel Ha near Tulum, Quintana Roo has approximately 100 km of surveyed passages and more than 50 known cenotes associated with it. There is uncertainty to what extent these cenote systems in the Yucatan Peninsula are connected. However, two biogeographic scenarios can be invoked to account the observed pattern of mtDNA types distribution in this region. Avise et al. (1987) defined major categories of possibilities towards intraspecific phylogenies in respect to their geographic domain. One category involved *phylogenetic continuity and spatial separation*. Accordingly, the geographic localization of closely related

genotypes, in the absence of major phylogenetic breaks, "involves historically limited gene flow between populations in species not subdivided by firm long-term zoogeographic barriers to dispersal". A second category involved *phylogenetic continuity and lack of spatial separation*. They proposed, "geographic populations of species exhibiting this category of intraspecific phylogeography have had relatively extensive and recent historical interconnections through gene flow". Due to the remarkably low amount of nucleotide divergence seen across *Typhlatya mitchelli* subpopulations and individuals (Table 14) and the uncertainty of the relationships that the geographically isolated cenotes share in which these shrimp were collected, either of the two categories seems plausible in describing the population biogeography of *Typhlatya mitchelli*. With the exception of the shrimp from Cenotes Kakuel and San Antonio Chiich (SAY), individual specimens only varied by one or two nucleotides in pairwise comparisons. However, these few mutations caused localizing of genotypes (again, refer to Table 13).

TABLE 14
Summary of Intraspecific Nucleotide Divergence Rates for *Typhlatya mitchelli*

	Among Subpopulations (range of Da)	Among Individuals (range of d)
Cytochrome *b*	0 – 0.31%	0 – 1.7%
Cytochrome Oxidase I	0 – 0.25%	0 – 1.3%
16S rRNA	0 – 0.21%	0 – 0.4%
Combined Sequences	0 – 0.13%	0 – 0.8%

Tempo and Mode of Molecular Evolution among Typhlatya

Palumbi and Benzie (1991) were the first to propose an accelerated rate of mtDNA nucleotide divergence in shrimp. They examined fragments of genes for 12S rRNA and COI between morphologically similar penaeid shrimp. Using only transversions, the subgenus *Litopenaeus* exhibited a 7.4% genetic differentiation level within a 700–800 bp portion of COI. Similarly, when looking at the current data using only transversions, *T. mitchelli* exhibited a 6.1% genetic differentiation level for a smaller fragment of COI when compared to *T. pearsei*. *Typhlatya mitchelli* varied from 2.8-3.0% from the new ESU from SAY and 5.7-6.4% when compared to *T. pearsei* using cytochrome *b* sequences, while 16S sequences revealed lower levels at 1.6% and 3.3% for the two respective comparisons. Evaluating the sequences with transitions and transversions, it does seem that there is an accelerated rate of nucleotide divergence among these shrimp similar to that reported for penaeid shrimp (Table 15). An alternative hypothesis proposed by Palumbi and Benzie is that a reduced rate of morphological change was occurring due to a stabilizing selection over an extended period of time. They also noted that cytochrome *b* is less conserved in amino acid sequence than cytochrome oxidase I. This study provides evidence that cytochrome *b* displays a faster rate of mutation in shrimp than COI.

TABLE 15
Summary of Interspecific Nucleotide Divergence Rates as Compared to *T. mitchelli*

	Cytochrome *b*	Cytochrome oxidase I	16S rRNA	Combined data
SAY shrimp	13.7 ± 0.2%	N/A	5.6 ± 0.1%	9.6%
T. pearsei	25.4 ± 0.6%	20.7 ± 0.3%	16.1 ± 0.3%	20.5 ± 0.3%

Boutin and Coineau (2000) suggested that fauna living in the subterranean realm for a long time (geologically speaking) exhibit a higher molecular polymorphism than epigean relatives. In a highly stable environment, successive generations gradually accumulate neutral mutations over the course of time. Although a higher rate of molecular evolution would exist, the rate of physiological and morphological evolution would be less than a related epigean taxon. If true, this would provide a similar, yet alternate means for witnessing high nucleotide divergence levels among stygobitic shrimp. If these hypotheses are correct, one can assume a similar divergence rate for *Typhlatya* as ones used in penaeid and other shrimp (2–3% per million years). Using the pairwise distances generated for the cytochrome *b* data, which showed the highest divergence levels, *T. mitchelli* and *T. pearsei* shared a common ancestor approximately 8.3–13.5 mya. Using the least divergent gene, 16S rRNA, the time is lowered to between 4.8–8.3 mya. Averaging all mitochondrial data would lead one to believe that the two species separated between 7–10 mya. *Typhlatya mitchelli* and the new *Typhlatya* group from San Antonio Chiich in Yokdzonot (SAY) last shared a common ancestor somewhere between 2–7 mya, using the low and high estimates of the two genes mentioned previously, with the average being 3–5 mya, during the mid-Pliocene era. *Typhlatya pearsei* is similar in percent divergence with respect to *T. mitchelli* and the new ESU from SAY, approximately 25% when looking at the cytochrome *b* data and around 16% for the 16S rRNA data. This suggests that the ancestor to the extant *T. mitchelli* and *T. pearsei* were isolated from each other some 7–10 mya and that the shrimp from SAY share common ancestry with the *T. mitchelli* species and that

somehow the ancestors became separated around 3–5 million years ago from a common gene pool. Due to logistical difficulties associated with the cenote SAY, a dive was not performed and all shrimp were collected while swimming in the entrance pool. Therefore, it is uncertain as to whether the cave has passages that are extensive and perhaps connected with the larger network of underwater passages. It can only be hypothesized that this cenote created a physical barrier to gene flow due to breakdown, lack of horizontal passages or perhaps horizontal passages that are deeper than the freshwater lens and therefore of no use to limnostygobites. This would be congruent with the geology of this part of the Yucatan Peninsula, which was formed out of Eocene deposits and is known to contain some of the larger and deeper cenotes on the peninsula (Reddell, 1977).

Numerous studies place the marine ancestor to *Typhlatya* sometime in the late Mesozoic (Monod and Cals, 1970; Sans and Platvoet, 1995). As Sans and Platvoet (1995) noted, if *Typhlatya* had a more recent evolution from a marine atyid then there would be a higher morphological similarity pattern among the geographically closer species. In addition, there are only a handful of anchialine animals related to marine taxa. Finally, seafloor dispersal would not be suitable for tropical/temperate fauna, only for cold-adapted species. Accordingly, Sans and Platvoet (1995) suggest that the common ancestor was split into three populations: European, Mid-Atlantic Ridge and Central American, due to the opening of Atlantic and an end to global Tethyan currents. Through further vicariant events, the Central American population was again subdivided. Isphording (1975) indicated that the two hill systems of Sierra de Ticul and

Sierra de Bolonchen formed a Miocene shoreline. Reddell's survey (1977) noted that this area contains thousands of caves. He also documented that *T. pearsei* only is found in this area and not *T. mitchelli*. A plausible scenario is the *T. pearsei* ancestor began to penetrate the brackish waters of this early Miocene shoreline, resulting in genetic isolation from the Central American ancestral stock. This is in agreement with the molecular results obtained in the current study. Using molecular clocks obtained in previous shrimp studies (Knowlton et al., 1993; Baldwin et al., 1998) based on mtDNA sequence divergence, *T. mitchelli* and *T. pearsei* last shared a common ancestor between 7–10 mya, sometime between the middle to late Miocene. Over time, with further lifting of the Yucatan and the subsequent development of elaborate karst systems, *T. pearsei* continued to expand its territory across the entire Yucatan Peninsula. It was during this same period, around the middle of the Pliocene, that the marine ancestor of *T. mitchelli* began moving into lower saline environments. Over time, successful colonization for both *T. pearsei* and *T. mitchelli* led to their acquiring a sympatric relationship in numerous cenotes across the region. The new ESU from Cenote San Antonio Chiich in Yokdzonot could possibly be explained by that particular cenote never being connected to the main network of subterranean systems and the founder population becoming isolated since being stranded by marine regression. It seems unlikely that this one cenote would not have ever been connected to such a massively, interconnected karst system. It is more likely that this cenote was colonized through active expansion. Later on, approximately 4 million years ago during the Pliocene, according to the interpretation of the molecular data, the SAY population became isolated from the

remainder of the gene pool, halting gene flow. A similar, but more recent scenario, could be invoked to explain the presence of numerous and distinct haplotypes at Cenote Kakuel. The higher levels of diversity observed in Cenote Kakuel may be due to a larger and more stable population (with larger Ne) than any other cave system surveyed in this study. However, due to the paucity of sampling across all caves, one cannot conclude which scenario best explains this apparent higher level of diversity.

Evolution of Anchialine Fauna and the Caves They Inhabit

The transition zone is produced where brackish and saline waters mix (Dodson, 2000). This transition zone is known as a halocline and is under saturated with respect to calcium and contains elevated amounts of carbon dioxide. This is significant because carbon dioxide then reacts with water to form carbonic acid, which in turn can react with the calcium thereby increasing the permeability of the bedrock in contact with the halocline. Additionally, over geological time, permeability increases progressively inland from the coast both vertically and horizontally due to sea level change, thus forming extensive karst systems (Moore and Sullivan, 1978). Depending on the position of the water table, caves are actively forming on top of or beneath old ones. Therefore, it is logical to believe that animals will move accordingly to occupy these newly developing living spaces (Holsinger, 1988; Iliffe, pers. comm.). This hypothesis is helpful in reinforcing an ancient colonization for many anchialine fauna, especially in a setting such as the Yucatan Peninsula where there is such a vast and complex subterranean network. Also lending support to the ancient colonization of certain anchialine fauna in the Yucatan is the presence of living fossils, or relics, such as the

crustacean class Remipedia (Yager, 1981). Remipedes are stygobites and therefore limited to caves; however, they have a highly disjunct distribution that includes islands in the Caribbean, the Canary Islands and Western Australia. This distribution tends to support a Tethyan origin where an ancestral species perhaps colonized coastal caves and later distributed through plate tectonic rafting. Subterranean Tethyan relicts have no congenerics in the open sea and appear in areas that were covered by the Eocene-Miocene seas (Por, 1986). As noted by Sans and Platvoet (1995), a new member of the genus *Typhlatya* was discovered in Spain, on the Mediterranean Sea coastline. Consequently, similar to other stygobites with highly disjunct distributions, this would lend support to a Tethyan origin for the ancestral stock of the extant *Typhlatya* members.

Multiple studies on stygofaunas have focused on the Late Tertiary, mostly Pleistocene, (Stock, 1986), but others have recognized the likely ancient vicariance in many stygofaunas (Rouch and Danielpol, 1987). The presence of congeneric species of cavernicoles on continental landmasses, as well as on islands on mid-oceanic ridges, has always presented problems in explaining the overall distribution of some anchialine fauna (Iliffe et al., 1983). Bermuda, the Bahamas, and Ascension Island, all being inhabited by different species of *Typhlatya*, have a common origin in the mid-Atlantic ridge in the late Cretaceous, about a 110 mya (Manning et al., 1986). These islands were never in contact with continental landmasses and therefore could not have been colonized by fauna restricted to tectonic rafts. A number of studies published concern the historical biogeography of different groups of marine stygobitic crustaceans in Central and North America (Stock, 1986; Holsinger, 1986). Conclusions of the studies

emphasizes that the origin of stygobitic lineages in continental groundwaters is more ancient than previously supposed, sometime during the Cretaceous and early Tertiary. However, the molecular data presented here indicates that the Caribbean ancestral stock to *Typhlatya* did not colonize the subterranean environment earlier than the mid-Tertiary.

CONCLUSION

In several crustacean studies, hydrologic relationships appear to reflect population connectivity and gene flow much better than geographic distance. In this study, I found overwhelming homogeneity of *T. mitchelli* across the entire Yucatan Peninsula. As in other studies, population samples from different caves belonging to a single groundwater aquifer may show no trace of genetic differentiation, suggesting that they belong to a single panmictic gene pool (Cobolli Sbordoni et al., 1990). Overlapping, or sympatric, sibling species such as *T. mitchelli* and *T. pearsei* can be explained for by secondary contact between populations that had evolved allopatrically in previously separated groundwater systems (Cobolli Sbordoni et al., 1990).

The finding of a single, continuous subterranean freshwater system sheds light on several important issues. First, these specimens have helped in establishing a better understanding of the anchialine cave population biogeography associated with the Yucatan Peninsula of Mexico. Even though this environment is organically deprived of nutrients, a diverse and abundant group of organisms are present there and have persisted for millions of years. Second, the unveiling of a single continuum becomes more important when dealing with persistent problems such as limestone quarrying, other various construction projects that are detrimental to the limestone platform and pollution (e.g. using the local cenote as a waste site). The magnitudes of these problems become so complex because they are not only destroying their immediate settings, but are also having a significantly larger impact on a more regional scale. Finally, injury to these beautiful and delicate ecosystems can disrupt the growing tourism industry that is

so vitally important to Mexico. Cenotes are an important part of the "Eco-Park" tourism industry that is rapidly increasing in this part of the world. The importance of these issues become apparent when one realizes that small-scale perturbations can and will have dramatic effects on the entire peninsula.

Future Directions

Future experiments that could enhance the results of this study and understanding biogeography of anchialine fauna in general might include: 1) Additional molecular phylogenetic analyses on Yucatan cave fauna to see if other freshwater animals are as successful as *Typhlatya* in colonizing this region. If these cave systems are connected throughout the Yucatan Peninsula, as suggested with this study, then other stygobitic fauna (e.g. mysids, thermosbaenaceans, amphipods) should produce similar results as seen here. 2) Collecting marine stygobites farther inland, below the freshwater lens, (assuming technology continues to improve and allows scientists to reach these deeper waters) to determine if there are any ancient morphotypes (or genotypes) that could provide more insight into the origins of the anchialine fauna. 3) Obtaining and sequencing additional *Typhlatya* species from other locations in an effort to understand the evolution of this genus and perhaps discover what led them to abandon their oceanic existence.

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APPENDIX A

Specialized cave-diving techniques were required for the thorough investigation of anchialine caves. A dive is considered a cave dive when one uses SCUBA under a naturally occurring rock ceiling. A standard set of five rules was adhered to during all cave dives. These rules were developed in response to early cave diving accidents and the mistakes that lead to victim fatalities. Prosser and Grey (1992) discuss the list of rules that follows in more detail: 1) Take the time to get the proper training and never exceed the level of that training. Properly trained divers should be able to handle any unforeseen circumstance that might arise during the course of the dive and take the appropriate action to resolve it safely. 2) Always use a continuous guideline from the cave entrance to any point in the dive. This rule will prevent the diver from getting lost in a cave system. 3) Turn the dive, or begin the exit, after consuming no more than one-third of the initial air supply. This will ensure that the diver and buddy have enough air should one have an out of air emergency, even at the deepest point of penetration, and need to share air. 4) Limit the dive to a maximum of 130 feet (40m) due to physiological factors, such as oxygen toxicity and nitrogen narcosis that make dives more complex beyond this depth. 5) All divers must carry at least three battery-powered lights.

APPENDIX B

Pairwise Distances among all *Typhlatya* Specimens using the Tamura-Nei Evolutionary Model for Nucleotides with the Pairwise Deletion Option in effect in the Program MEGA

N2																	
CW2	0.000																
CW3	0.000	0.000															
CW4	0.000	0.000	0.000														
CW5	0.000	0.000	0.000	0.000													
CJ2	0.002	0.002	0.002	0.002	0.002												
CJ3	0.002	0.002	0.002	0.002	0.002	0.000											
CJ5	0.002	0.002	0.002	0.002	0.002	0.000	0.000										
CJ6	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.000									
CJ7	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.000								
CJ8	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.000	0.000							
SAC2	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.002	0.002	0.002	0.002						
SAC3	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.002	0.002	0.002	0.002	0.000					
SJ1	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.002				
SJ2	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.002			
SJ3	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.002	0.000		
SJ4	0.002	0.002	0.002	0.002	0.002	0.004	0.004	0.004	0.004	0.004	0.004	0.002	0.002	0.004	0.002	0.002	
SJ11	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.000	0.002	0.002	0.004
SJ12	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.000	0.002	0.002	0.004
SJ14	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.000	0.002	0.002	0.004
K2	0.002	0.002	0.002	0.002	0.002	0.004	0.004	0.004	0.004	0.004	0.004	0.002	0.002	0.004	0.002	0.002	0.004
K3	0.010	0.010	0.010	0.010	0.010	0.013	0.013	0.013	0.013	0.013	0.013	0.010	0.010	0.013	0.010	0.010	0.013
K4	0.004	0.004	0.004	0.004	0.004	0.006	0.006	0.006	0.006	0.006	0.006	0.004	0.004	0.006	0.004	0.004	0.006
K5	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.017
K6	0.002	0.002	0.002	0.002	0.002	0.004	0.004	0.004	0.004	0.004	0.004	0.002	0.002	0.004	0.002	0.002	0.004
SAY2	0.135	0.135	0.135	0.135	0.135	0.138	0.138	0.138	0.138	0.138	0.138	0.135	0.135	0.138	0.135	0.135	0.138
SAY3	0.135	0.135	0.135	0.135	0.135	0.138	0.138	0.138	0.138	0.138	0.138	0.135	0.135	0.138	0.135	0.135	0.138
SAY4	0.135	0.135	0.135	0.135	0.135	0.138	0.138	0.138	0.138	0.138	0.138	0.135	0.135	0.138	0.135	0.135	0.138
SAY5	0.135	0.135	0.135	0.135	0.135	0.138	0.138	0.138	0.138	0.138	0.138	0.135	0.135	0.138	0.135	0.135	0.138
SAY6	0.135	0.135	0.135	0.135	0.135	0.138	0.138	0.138	0.138	0.138	0.138	0.135	0.135	0.138	0.135	0.135	0.138
AA3	0.248	0.248	0.248	0.248	0.248	0.251	0.251	0.251	0.251	0.251	0.251	0.248	0.248	0.251	0.248	0.248	0.245
27-1p	0.260	0.260	0.260	0.260	0.260	0.263	0.263	0.263	0.263	0.263	0.263	0.260	0.260	0.263	0.260	0.260	0.257
SM6	0.249	0.249	0.249	0.249	0.249	0.252	0.252	0.252	0.252	0.252	0.252	0.249	0.249	0.252	0.249	0.249	0.246
	N2	CW2	CW3	CW4	CW5	CJ2	CJ3	CJ5	CJ6	CJ7	CJ8	SAC2	SAC3	SJ1	SJ2	SJ3	SJ4

	SJ11	SJ12	SJ14	K2	K3	K4	K5	K6	SAY2	SAY3	SAY4	SAY5	SAY6	AA3	27-1p	SM6
SJ11																
SJ12	0.000															
SJ14	0.000	0.000														
K2	0.004	0.004	0.004													
K3	0.013	0.013	0.013	0.013												
K4	0.006	0.006	0.006	0.006	0.010											
K5	0.015	0.015	0.015	0.017	0.004	0.015										
K6	0.004	0.004	0.004	0.004	0.013	0.006	0.017									
SAY2	0.138	0.138	0.138	0.138	0.133	0.135	0.138	0.138								
SAY3	0.138	0.138	0.138	0.138	0.133	0.135	0.138	0.138	0.000							
SAY4	0.138	0.138	0.138	0.138	0.133	0.135	0.138	0.138	0.000	0.000						
SAY5	0.138	0.138	0.138	0.138	0.133	0.135	0.138	0.138	0.000	0.000	0.000					
SAY6	0.138	0.138	0.138	0.138	0.133	0.135	0.138	0.138	0.000	0.000	0.000	0.000				
AA3	0.251	0.251	0.251	0.245	0.253	0.248	0.259	0.251	0.254	0.254	0.254	0.254	0.254			
27-1p	0.263	0.263	0.263	0.256	0.265	0.260	0.271	0.263	0.264	0.264	0.264	0.264	0.264	0.021		
SM6	0.252	0.252	0.252	0.245	0.254	0.249	0.259	0.252	0.242	0.242	0.242	0.242	0.242	0.015	0.023	

APPENDIX C

COI Pairwise Distances Performed in MEGA, Utilizing the Tamura-Nei Nucleotide Evolutionary Model and the Pairwise Deletion Option Selected

	N2	CW2	CW3	CW4	CJ2	CJ3	CJ5	SAC2	SAC3	SJ1	SJ2	SJ3	K2	K3	K4	AA3	27-1p	SM6
N2																		
CW2	0.002																	
CW3	0.002	0.000																
CW4	0.002	0.000	0.000															
CJ2	0.002	0.000	0.000	0.000														
CJ3	0.002	0.000	0.000	0.000	0.000													
CJ5	0.002	0.000	0.000	0.000	0.000	0.000												
SAC2	0.002	0.000	0.000	0.000	0.000	0.000	0.000											
SAC3	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000										
SJ1	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000									
SJ2	0.004	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002								
SJ3	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002							
K2	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000						
K3	0.013	0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.009	0.011	0.011					
K4	0.004	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.009				
AA3	0.206	0.203	0.203	0.203	0.203	0.203	0.203	0.203	0.203	0.203	0.203	0.203	0.203	0.203	0.203			
27-1p	0.211	0.208	0.208	0.208	0.208	0.208	0.208	0.208	0.208	0.208	0.208	0.208	0.208	0.208	0.208	0.006		
SM6	0.207	0.210	0.210	0.210	0.210	0.210	0.210	0.210	0.210	0.210	0.210	0.210	0.210	0.210	0.210	0.021	0.023	

APPENDIX D

Pairwise Distances of 16S rRNA Sequences as Performed in MEGA

N2																								
CW2	0.002																							
CW3	0.000	0.002																						
CW4	0.000	0.002	0.000																					
CJ2	0.000	0.002	0.000	0.000																				
CJ3	0.000	0.002	0.000	0.000	0.000																			
CJ5	0.000	0.002	0.000	0.000	0.000	0.000																		
SAC2	0.002	0.004	0.002	0.002	0.002	0.002	0.002																	
SAC3	0.002	0.004	0.002	0.002	0.002	0.002	0.002	0.000																
SJ1	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.002															
SJ2	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.000														
SJ3	0.002	0.004	0.002	0.002	0.002	0.002	0.002	0.000	0.000	0.002	0.002													
K2	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.000	0.000	0.002												
K3	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.000	0.000	0.002	0.000											
K4	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.000	0.000	0.002	0.000	0.000										
SAY2	0.056	0.058	0.056	0.056	0.056	0.056	0.056	0.059	0.059	0.056	0.056	0.059	0.056	0.056	0.056									
SAY3	0.056	0.058	0.056	0.056	0.056	0.056	0.056	0.059	0.059	0.056	0.056	0.059	0.056	0.056	0.056	0.000								
SAY4	0.054	0.056	0.054	0.054	0.054	0.054	0.054	0.056	0.056	0.054	0.054	0.056	0.054	0.054	0.054	0.002	0.002							
SAY5	0.056	0.058	0.056	0.056	0.056	0.056	0.056	0.059	0.059	0.056	0.056	0.059	0.056	0.056	0.056	0.000	0.000	0.002						
SAY6	0.056	0.058	0.056	0.056	0.056	0.056	0.056	0.059	0.059	0.056	0.056	0.059	0.056	0.056	0.056	0.000	0.000	0.002	0.000					
AA3	0.162	0.162	0.162	0.162	0.162	0.162	0.162	0.159	0.159	0.162	0.162	0.159	0.162	0.162	0.162	0.148	0.148	0.146	0.148	0.148				
27-1p	0.165	0.165	0.165	0.165	0.165	0.165	0.165	0.162	0.162	0.165	0.165	0.162	0.165	0.165	0.165	0.151	0.151	0.149	0.151	0.151	0.002			
SM6	0.159	0.158	0.159	0.159	0.159	0.159	0.159	0.156	0.156	0.159	0.159	0.156	0.159	0.159	0.159	0.145	0.145	0.143	0.145	0.145	0.006	0.004		
	N2	CW2	CW3	CW4	CJ2	CJ3	CJ5	SAC2	SAC3	SJ1	SJ2	SJ3	K2	K3	K4	SAY2	SAY3	SAY4	SAY5	SAY6	AA3	27-1p	SM6	

APPENDIX E

Cytochrome *b* sequences

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                                                                      45
N2    C TTT GTT TGT TTA TAT ATA CAT ATT GGA CGT GGG ATC TAC TAT GG
CW2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW4   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW5   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ5   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ6   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ7   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ8   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAC2  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAC3  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ1   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ4   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ11  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ12  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ14  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K2    . ... ... ... ... ..C ... ... ... ... ... ... ... ... ... ..
K3    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K4    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K5    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K6    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAY2  T ... ..C ..C ..G ... ..G ... G.A .A. ... ..C ... ... .T. ..
SAY3  T ... ..C ..C ..G ... ..G ... G.A .A. ... ..C ... ... .T. ..
SAY4  T ... ..C ..C ..G ... ..G ... G.A .A. ... ..C ... ... .T. ..
SAY5  T ... ..C ..C ..G ... ..G ... G.A .A. ... ..C ... ... .T. ..
SAY6  T ... ..C ..C ..G ... ..G ... G.A .A. ... ..C ... ... .T. ..
AA3   T G.. ... ..C ... ..C ... ... G.A ... ..C ..A ... ... ... ..
27-1p T ... ... ..C ... ..C ... ... G.A ... ..C ..A ... ... ... ..
SM6   T ... ... ..C ... ..C ... ..C G.A ... ..C ..A ... ... ... ..

                                                                      90
N2    A TCT TTC TTA TTT TTA CAT ACA TGA ATA ATT GGA GTT GTA ATT CT
CW2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW4   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW5   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ5   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ6   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ7   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ8   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAC2  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAC3  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
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SJ1   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ4   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ11  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ12  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ14  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K2    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K3    . ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ... ..
K4    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K5    . ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ... ..
K6    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAY2  . ..C ... ... ... C.. ... ... ..G ... G.. ... ... ... ... ..
SAY3  . ..C ... ... ... C.. ... ... ..G ... G.. ... ... ... ... ..
SAY4  . ..C ... ... ... C.. ... ... ..G ... G.. ... ... ... ... ..
SAY5  . ..C ... ... ... C.. ... ... ..G ... G.. ... ... ... ... ..
SAY6  . ..C ... ... ... C.. ... ... ..G ... G.. ... ... ... ... ..
AA3   . ..A ..T ... .AC ACC ..C ... ... C.. G.A ... ..C ... ... ..
27-1p . ..A ..T ..G .AC ACC ..C ... ... C.. G.A ... ..C ... ... ..
SM6   . ..A ..T ... .AC ACC ..C ... ... C.. G.A ... ..C ... ... ..

                                                                 135
N2    T CTT TTA ACT ATA GCA ACA GCC TTT CTA GGA TAT GTT CTT CCG TG
CW2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW4   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW5   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ5   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ6   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ7   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ8   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAC2  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAC3  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ1   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ4   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ11  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ12  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ14  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K2    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K3    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K4    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K5    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K6    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAY2  C ... ... ... ... ..G ... ... ..C T.G ... ... ... ..C ..A ..
SAY3  C ... ... ... ... ..G ... ... ..C T.G ... ... ... ..C ..A ..
SAY4  C ... ... ... ... ..G ... ... ..C T.G ... ... ... ..C ..A ..
SAY5  C ... ... ... ... ..G ... ... ..C T.G ... ... ... ..C ..A ..
SAY6  C ... ... ... ... ..G ... ... ..C T.G ... ... ... ..C ..A ..
AA3   C A.C C.. ... ... ... ..C ..T ..C ... ..C ... ..C ..C ... ..
27-1p C A.C C.. ... ... ... ..C ..T ..C ... ..C ... ..C ..C ... ..
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SM6   C A.C C.. ... ... ... ..C ..T ..C ... ..C ... ..C ..C ... ..

                                                                  180
N2    A GGA CAA ATA TCT TTC TGG GGA GCT ACA GTA ATT ACT AAT CTA TT
CW2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW4   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW5   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ5   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ6   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ7   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ8   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAC2  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAC3  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ1   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ4   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ11  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ12  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ14  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K2    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K3    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K4    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K5    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K6    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAY2  . ... ... ... ... ..T ..A ... ..G ... ..G ... ... ... T.. ..
SAY3  . ... ... ... ... ..T ..A ... ..G ... ..G ... ... ... T.. ..
SAY4  . ... ... ... ... ..T ..A ... ..G ... ..G ... ... ... T.. ..
SAY5  . ... ... ... ... ..T ..A ... ..G ... ..G ... ... ... T.. ..
SAY6  . ... ... ... ... ..T ..A ... ..G ... ..G ... ... ... T.. ..
AA3   . ... ... ... ..C ... ..A ... ..C ... ... ... ... ... T.. ..
27-1p . ... ... ... ..C ... ... ... ..C ... ... ... ... ... T.. ..
SM6   . ... ... ... ..C ... ..A ... ..C ... ... ... ... ... T.. ..

                                                                  225
N2    T TCT ACT ATT CCC TAT ATT GGA TCT GAC TTT GTT CAA TGA ATT TG
CW2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW4   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW5   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ5   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ6   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ7   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ8   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAC2  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAC3  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ1   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
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SJ4   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ11  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ12  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ14  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K2    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K3    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K4    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K5    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K6    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAY2  . ..A ... ..C ..A ... ... ..G ... ..T ... ... ... ... ... ... ..
SAY3  . ..A ... ..C ..A ... ... ..G ... ..T ... ... ... ... ... ... ..
SAY4  . ..A ... ..C ..A ... ... ..G ... ..T ... ... ... ... ... ... ..
SAY5  . ..A ... ..C ..A ... ... ..G ... ..T ... ... ... ... ... ... ..
SAY6  . ..A ... ..C ..A ... ... ..G ... ..T ... ... ... ... ... ... ..
AA3   . ..A ..C ..C ... ... ..C ... C.A ..T ..C ... ... ... ... ... ..
27-1p . ..A ..C ..C ... ... ..C ... C.A ..T ..C ... ... ... ... ... ..
SM6   . ..A ..C ..C ... ... ..C ... C.A ..T ..C ... ... ... ... ... ..

                                                                   270
N2    A GGA GGA TTC GCA GTA GAT AAT GCC ACC CTC ACC CGG TTT TAT GC
CW2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW4   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW5   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ5   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ6   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ7   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ8   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAC2  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAC3  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ1   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ4   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ11  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ12  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ14  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K2    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K3    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K4    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K5    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K6    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAY2  . ... ... ..T ... ..T ... ... ... ... ..T ... ..A ... ... ... ..
SAY3  . ... ... ..T ... ..T ... ... ... ... ..T ... ..A ... ... ... ..
SAY4  . ... ... ..T ... ..T ... ... ... ... ..T ... ..A ... ... ... ..
SAY5  . ... ... ..T ... ..T ... ... ... ... ..T ... ..A ... ... ... ..
SAY6  . ... ... ..T ... ..T ... ... ... ... ..T ... ..A ... ... ... ..
AA3   . ..G ..C ..T ... ... ... .GC ..T ... T.A ... ... ..C .T. ..
27-1p . ..G ..C ..T ... ... ... .GC ..T ... T.A ... ... ..C .T. ..
SM6   . ..G ..C ..T ... ... ... .G. ..T ... T.A ... ... ..C .T. ..
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                                                                                                                                315
N2     G TTC CAT TTC CTT TTT CCA TTT GCA ATT ATA GCT GCC TCT ATA AT
CW2    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW3    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW4    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW5    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ2    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ3    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ5    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ6    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ7    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ8    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAC2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAC3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ1    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ2    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ3    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ4    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ11   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ12   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ14   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K2     . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K3     A ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ..
K4     . ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ..
K5     A ... ... ... ... ... ... ... .T. ... ... ... A.. ... ... ..
K6     . ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ..
SAY2   A ... ... ... ..C ... ... ..C ... ... ... ... ... ... ... G.
SAY3   A ... ... ... ..C ... ... ..C ... ... ... ... ... ... ... G.
SAY4   A ... ... ... ..C ... ... ..C ... ... ... ... ... ... ... G.
SAY5   A ... ... ... ..C ... ... ..C ... ... ... ... ... ... ... G.
SAY6   A ... ... ... ..C ... ... ..C ... ... ... ... ... ... ... G.
AA3    A ... ..C ... A.. C.. ..G ... ... .CC ... ..A ..A ..C ... G.
27-1p  A ... ..C ... A.. C.. ..G ..C ... .CC ... ..A ..A ..C ... G.
SM6    A ... ..C ... G.. C.. ..G ..C ... .CC ... ..A ..A ..C ... G.

                                                                                                                                360
N2     C CAT ATC CTG TTC CTT CAC CAG ACA GGA TCA AAC AAC CCC CTA GG
CW2    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW3    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW4    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW5    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ2    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ3    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ5    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ6    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ7    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ8    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAC2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAC3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ1    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ2    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ3    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ4    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ11   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
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SJ12  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ14  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K2    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K3    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K4    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K5    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K6    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAY2  G ... ..T T.A ..T ..A ... ..A ... ... ... ... ... ... T.. ..
SAY3  G ... ..T T.A ..T ..A ... ..A ... ... ... ... ... ... T.. ..
SAY4  G ... ..T T.A ..T ..A ... ..A ... ... ... ... ... ... T.. ..
SAY5  G ... ..T T.A ..T ..A ... ..A ... ... ... ... ... ... T.. ..
SAY6  G ... ..T T.A ..T ..A ... ..A ... ... ... ... ... ... T.. ..
AA3   . ... ... ... ... ..C ..T ..A ... ... ... ... ..T ... ... ..
27-1p . ... ... ... ... ..C ..T ..A ... ... ... ... ..T ... ... ..
SM6   . ... ... ..A ... ..C ..T ..A ... ... ... ... ..T ... ... ..

                                                                 405
N2    A GTT TTA TCC CAT ATC AAT AAA ATT CCA TTC CAC CCA TAT TTT TC
CW2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW4   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW5   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ5   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ6   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ7   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ8   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAC2  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAC3  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ1   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ4   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ11  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ12  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ14  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K2    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K3    . ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ..
K4    . ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ..
K5    . ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ..
K6    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAY2  . A.. G.T ... ..A ..T ... ... G.. ... ... ... ... ... ..C ..
SAY3  . A.. G.T ... ..A ..T ... ... G.. ... ... ... ... ... ..C ..
SAY4  . A.. G.T ... ..A ..T ... ... G.. ... ... ... ... ... ..C ..
SAY5  . A.. G.T ... ..A ..T ... ... G.. ... ... ... ... ... ..C ..
SAY6  . A.. G.T ... ..A ..T ... ... G.. ... ... ... ... ... ..C ..
AA3   G A.C CCG ..G ..A .C. ... ... G.A ..G ... ... ..T ..C ..C ..
27-1p G A.C CCG C.A ..A .C. ... ... G.A ..G ... ... ..T ..C ..C ..
SM6   G A.C CCG ..G ..A .C. ... ... G.A ..G ... ... ..T ..C ..C ..

                                                                 450
N2    C TTT AAA GAT ATT GTA GGA TTT ATT ATT TTA CTT TTA ATA TTA GC
CW2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
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CW3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW4   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CW5   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
CJ2   . ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ..
CJ3   . ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ..
CJ5   . ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ..
CJ6   . ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ..
CJ7   . ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ..
CJ8   . ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ..
SAC2  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAC3  . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ1   . ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ..
SJ2   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ3   . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SJ4   . ... ... ... ... ... ... ... ... ... ... ... ... ... G.. ... ..
SJ11  . ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ..
SJ12  . ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ..
SJ14  . ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ..
K2    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K3    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K4    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
K5    . ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ... ..
K6    . ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
SAY2  . ... ... ... ... ... ... ... ... ... ... ... ... ... .C. ... ..
SAY3  . ... ... ... ... ... ... ... ... ... ... ... ... ... .C. ... ..
SAY4  . ... ... ... ... ... ... ... ... ... ... ... ... ... .C. ... ..
SAY5  . ... ... ... ... ... ... ... ... ... ... ... ... ... .C. ... ..
SAY6  . ... ... ... ... ... ... ... ... ... ... ... ... ... .C. ... ..
AA3   . ..C ... ... ... ... ... ..C .G. ..C .CC ... ... GCC ... ... ..
27-1p . ..C ... ... ... ... ... ..C G.C ..C .CC ... ... GCC ... ... ..
SM6   . ..C ... ... ... ... ... ..C ... ..C .CC ... ... GCC ... ... ..

                                                        483
N2    A ATA ATC ACC TTA CTT TTC CCG TAT CTC TTA AG
CW2   . ... ... ... ... ... ... ... ... ... ... ..
CW3   . ... ... ... ... ... ... ... ... ... ... ..
CW4   . ... ... ... ... ... ... ... ... ... ... ..
CW5   . ... ... ... ... ... ... ... ... ... ... ..
CJ2   . ... ... ... ... ... ... ... ... ... ... ..
CJ3   . ... ... ... ... ... ... ... ... ... ... ..
CJ5   . ... ... ... ... ... ... ... ... ... ... ..
CJ6   . ... ... ... ... ... ... ... ... ... ... ..
CJ7   . ... ... ... ... ... ... ... ... ... ... ..
CJ8   . ... ... ... ... ... ... ... ... ... ... ..
SAC2  . ... ... ... ... ... ... ... ... ... ... ..
SAC3  . ... ... ... ... ... ... ... ... ... ... ..
SJ1   . ... ... ... ... ... ... ... ... ... ... ..
SJ2   . ... ... ... ... ... ... ... ... ... ... ..
SJ3   . ... ... ... ... ... ... ... ... ... ... ..
SJ4   . ... ... ... ... ... ... ... ... ... ... ..
SJ11  . ... ... ... ... ... ... ... ... ... ... ..
SJ12  . ... ... ... ... ... ... ... ... ... ... ..
SJ14  . ... ... ... ... ... ... ... ... ... ... ..
K2    . ... ... ... ... ... ... ... ... ... ... ..
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K3    . ... ... ... ... ... ... ..A ... ... ... ..
K4    . ... ... ... ... ... ... ... ... ... ... ..
K5    . ... ... ... ... ... ... ..A ... ... ... ..
K6    . ... ... ... ... ... ... ... ... ... ... ..
SAY2  . ... ... ... ... ... .A. ..A ... ..T ... ..
SAY3  . ... ... ... ... ... .A. ..A ... ..T ... ..
SAY4  . ... ... ... ... ... .A. ..A ... ..T ... ..
SAY5  . ... ... ... ... ... .A. ..A ... ..T ... ..
SAY6  . ... ... ... ... ... .A. ..A ... ..T ... ..
AA3   . ... ..T ... C.. ... .A. ..C ..C ... C.. ..
27-1p . ... ..T ... C.. ... CA. ..C ..C ... C.. ..
SM6   . ... ..T ... C.. ... .A. ..C ..C ... C.. ..
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APPENDIX F

Cytochrome Oxidase I

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                                                                      45
N2   CC CAC ATT GTC AGA CAA GAA TCA AGA AAA AAA GAA ACC TTT GGT A
CW2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW4  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ5  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC2 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC3 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ1  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K2   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K3   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K4   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
AA3  .. ..T ... ..A ... ... ... ..T ... ... ... ... ... ..C ..C .
271P .. ..T ... ..A ... ... ... ..T ... ... ... ... ... ..C ..C .
SM6  .. ..T ... ..A ... ... ... ..T ... ... ... ... ... ..C ..C .

                                                                      90
N2   CC TTA GGT ATA GTG TAT GCT ATA ATA GCA ATT GGT ATT CTA GGG T
CW2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW4  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ5  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC2 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC3 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ1  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K2   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K3   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ..A .
K4   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
AA3  .G C.. ..C ..G ..A ..C ..C ... ... ... ..C ..A ... T.. ..C .
271P .G C.. ..C ..G ..A ..C ..C ... ... ... ..C ..A ... T.. ..C .
SM6  .G C.. ..C ..G ..A ..C ..C ... ... ... ..C ..A ... T.. ..C .

                                                                     135
N2   TT GTT GTA TGA GCT CAC CAT ATA TTT ACC GTA GGT CTT GAT GTT G
CW2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW4  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
J2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ5  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC2 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
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SAC3 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ1  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K2   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K3   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K4   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
AA3  .C A.C ..C ... ..C ... ... ... ..C ..A ... ..A ..A ... ... .
271P .C A.C ..C ... ..C ... ... ... ..C ..A ... ..A ..A ... ... .
SM6  .C A.C ..C ... ... ... ... ... ..C ..A ... ..C ..G ... ... .

                                                              180
N2   AT ACC CGA GCC TAC TTT ACT TCA GCC ACA ATA ATT ATT GCT GTT C
CW2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW4  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ5  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC2 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC3 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ1  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K2   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K3   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K4   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
AA3  .C ..A ... ... ... ... ..C ... ... ... ... ... ... ... ..G .
271P .C ..A ... ... ... ... ..C ... ... ... ... ... ... ... ..G .
SM6  .C ..A ... ... ... ... ..C ... ... ... ... ... ... ... ..G .

                                                              225
N2   CA ACA GGA ATT AAA ATC TTT AGA TGG TTA GGG ACA CTT CAT GGT A
CW2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW4  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ5  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC2 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC3 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ1  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K2   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K3   .. ... ... ... ... ..T ... ... ... ... ..A ... ... ... ... .
K4   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
AA3  .. ... ... ..C ... ... ... ... ... C.. ..A ... ..C ..C ... .
271P .. ... ... ..C ... ... ... ... ..A C.. ..A ... ..C ..C ... .
SM6  .. ... ... ..C ... ... ... ... ... C.. ..A ... ..C ..C ... .

                                                              270
N2   AC AAA TTT AAC TAC AGG CCT TCC TTA TTA TGA GCC TTA GGA TTT A
CW2  .T ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
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CW3  .T ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW4  .T ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ2  .T ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ3  .T ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ5  .T ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC2 .T ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC3 .T ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ1  .T ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ2  .T ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ3  .T ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K2   .T ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K3   .T ... ... ... ... ... ... ... ... C.. ... ... ... ... ... .
K4   .T ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
AA3  .T ... ... .C. ... ..C ..A ... C.C C.. ... ... ... ..G ... .
271P .T ... ... .C. ... ..C ..A ... C.C C.. ... ... ... ..G ... .
SM6  .. ... ... .C. ... ..C ..A ... C.C C.. ... ... ... ..G ... .

                                                              315
N2   TC TTC CTC TTT ACT ATT GGA GGA CTC ACC GGC GTA ATT CTT TCA A
CW2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW4  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ5  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC2 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC3 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ1  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ2  .. ... ... ... ... ... ... ... ... ... ..T ... ... ... ... .
SJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K2   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K3   .. ... ... ... ... ... ... ... ... ... ..T ... ... ... ... .
K4   .. ... ... ... ... ... ... ... ... ... ..T ... ... ... ... .
AA3  .. ... T.A ... ..A ... ..G ... ..A ..A ..A ... G.. ..A ... .
271P .. ... T.A ... ..A ... ..G ... ..A ..A ..A ... G.. ..A ... .
SM6  .. ... T.A ... ..A ... ... ... ..A ..A ..A ... G.C ..A ... .

                                                              360
N2   AT TCA TCT ATT GAT ATT ATA CTC CAT GAT ACA TAT TAT GTT GTA G
CW2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW4  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ5  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC2 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC3 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ1  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K2   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K3   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K4   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
AA3  .C ..C ... ..C ..C ... ... ... ... ..C ... ... ... ..C ... .
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271P .C ..C ... ..C ..C ... ... ... ... ..C ... ... ... ..C ... .
SM6  .C ..C ... ..C ..C ... ... ... ... ..C ... ... ... ..C ... .

                                                              405
N2   CA CAT TTT CAC TAT GTT CTT TCA ATA GGT GCA GTC TTC GGA ATC T
CW2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW4  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ5  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC2 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC3 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ1  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K2   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K3   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K4   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
AA3  .C ..C ..C ... ..C ..C ..A ... ... ..A ... ..A ... ..G ..T .
271P .C ..C ..C ... ..C ..C ..A ... ... ..A ... ..A ... ..G G.T .
SM6  .C ..C ..C ... ..C ..C ..A ... ... ..G ... ..A ... ..G ..T .

                                                              450
N2   TT GCG GGA ATT GCT CAT TGG TTT CCG TTA TTT ACA GGG ATA ACA C
CW2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW4  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ5  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC2 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC3 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ1  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K2   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K3   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K4   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
AA3  .C ..C ..G ... ..C ..C ... ..C ..C C.. ... ... ... G.G ..C .
271P .C ..C ..G ... ..C ..C ... ..C ..C C.. ... ... ... G.G ..C .
SM6  .C ..C ..G ... ..C ..C ... ..C ..T C.. ... ... ... G.G ..C .

                                                              495
N2   TT CTT CCC AAA TGA CTC AAA ATT CAC TTC CTA ACT ATA TTT ATT A
CW2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW4  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ5  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC2 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC3 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ1  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
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SJ2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K2   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K3   .. ... ... ..G ... ... ... ... ... ... ... ... ... ... ... .
K4   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
AA3  .. .GC ... ... ... ..T ... ... ... ... ... ..C ... ..C ... .
271P .. .AC ... ... ... ..T ... ... ... ... ... ..C ... ..C ... .
SM6  .. .AC ... ... ... ..T ... ... ..T ... ... ..C ... ..C ... .

                                                                 540
N2   GA GTT AAT GCT ACA TTC TTT CCC CAG CAT TTC TTA GGT CTC AAC G
CW2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CW4  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
CJ5  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC2 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SAC3 .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ1  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ2  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
SJ3  .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K2   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K3   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
K4   .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
AA3  .. ... ... AT. ... ... C.. ..T ... ..C ... C.. ..C ..T ..T .
271P .. ... ... AT. ... ... C.. ..T ... ..C ... C.. ..C ..T ..T .
SM6  .. ... ... ATG ... ... C.. ..T ... ..C ... C.. ..C ..T ..T .

    542
N2   GT
CW2  ..
CW3  ..
CW4  ..
CJ2  ..
CJ3  ..
CJ5  ..
SAC2 ..
SAC3 ..
SJ1  ..
SJ2  ..
SJ3  ..
K2   ..
K3   ..
K4   ..
AA3  .A
271P .A
SM6  .A
```

APPENDIX G

16S rRNA sequences

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                                                                                                  60
N2   GAACAGACCT ACAATCAAGA CCCCTACACC TTGATGAAAT CTTAATTCAA CATCGAGGTC
CW2  .......... .......... .......... .......... .......... ..........
CW3  .......... .......... .......... .......... .......... ..........
CW4  .......... .......... .......... .......... .......... ..........
CJ2  .......... .......... .......... .......... .......... ..........
CJ3  .......... .......... .......... .......... .......... ..........
CJ5  .......... .......... .......... .......... .......... ..........
SAC2 .......... .......... .......... .......... .......... ..........
SAC3 .......... .......... .......... .......... .......... ..........
SJ1  .......... .......... .......... .......... .......... ..........
SJ2  .......... .......... .......... .......... .......... ..........
SJ3  .......... .......... .......... .......... .......... ..........
K2   .......... .......... .......... .......... .......... ..........
K3   .......... .......... .......... .......... .......... ..........
K4   .......... .......... .......... .......... .......... ..........
SAY2 .......... .........G .TA....... ..A....... .......... ..........
SAY3 .......... .........G .TA....... ..A....... .......... ..........
SAY4 .......... .......... .TA....... ..A....... .......... ..........
SAY5 .......... .........G .TA....... ..A....... .......... ..........
SAY6 .......... .........G .TA....... ..A....... .......... ..........
AA3  .......... ....G..... .TT..G..T. .........C .......... ..........
271P .......... ....G..... .TT..G..T. .........C .......... ..........
SM6  .......... ....G..... .TT..G..T. .........C .......... ..........

                                                                                                 120
N2   GCAAATACTT TTGTCGATAA GAACTCTCAA AAAGAATTAC GCTGTTATCC CTGGAGTAAC
CW2  .......... .......... .......... .......... .......... ..........
CW3  .......... .......... .......... .......... .......... ..........
CW4  .......... .......... .......... .......... .......... ..........
CJ2  .......... .......... .......... .......... .......... ..........
CJ3  .......... .......... .......... .......... .......... ..........
CJ5  .......... .......... .......... .......... .......... ..........
SAC2 .......... .......... .......... .......... .......... ..........
SAC3 .......... .......... .......... .......... .......... ..........
SJ1  .......... .......... .......... .......... .......... ..........
SJ2  .......... .......... .......... .......... .......... ..........
SJ3  .......... .......... .......... .......... .......... ..........
K2   .......... .......... .......... .......... .......... ..........
K3   .......... .......... .......... .......... .......... ..........
K4   .......... .......... .......... .......... .......... ..........
SAY2 .......... C......... .......... .......... .......... ..........
SAY3 .......... C......... .......... .......... .......... ..........
SAY4 .......... C......... .......... .......... .......... ..........
SAY5 .......... C......... .......... .......... .......... ..........
SAY6 .......... C......... .......... .......... .......... ..........
AA3  .......T.. C......... .......... G..A...... .......... ..........
271P .......T.. C......... .......... G..A...... .......... ..........
SM6  .......T.. C......... .......... G..A...... .......... ..........
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                                                                                                                  180
N2   TTAAATCTTA TAATCTATAA TACAAGATCA ACA-CCTAAA AATTATTTAT TTTACTATTA
CW2  .......... .......... .......... ...-...... .......... ..........
CW3  .......... .......... .......... ...-...... .......... ..........
CW4  .......... .......... .......... ...-...... .......... ..........
CJ2  .......... .......... .......... ...-...... .......... ..........
CJ3  .......... .......... .......... ...-...... .......... ..........
CJ5  .......... .......... .......... ...-...... .......... ..........
SAC2 .......... .......... .......... ...-...... .......... ..........
SAC3 .......... .......... .......... ...-...... .......... ..........
SJ1  .......... .......... .......... ...-...... .......... ..........
SJ2  .......... .......... .......... ...-...... .......... ..........
SJ3  .......... .......... .......... ...-...... .......... ..........
K2   .......... .......... .......... ...-...... .......... ..........
K3   .......... .......... .......... ...-...... .......... ..........
K4   .......... .......... .......... ...-...... .......... ..........
SAY2 .......... .....CG... .......... ...-...... .......... ..........
SAY3 .......... .....CG... .......... ...-...... .......... ..........
SAY4 .......... .....CG... .......... ...-...... .......... ..........
SAY5 .......... .....CG... .......... ...-...... .......... ..........
SAY6 .......... .....CG... .......... ...-...... .......... ..........
AA3  .......... .....C.C.. C...G..... .T.C...... ...C.C.... .......AC.
271P .......... .....C.C.. C...G..... .T.C...... ...C.C.... .......AC.
SM6  .......... .....C.C.. C...G..... .T.C...... ...C.C.... .......AC.

                                                                                                                  240
N2   AAAACAGTTA ATAATATATT TTAATCGCCC CAACCTAATT TTTATTAAAC TCAAATTTAA
CW2  .......... .......... .......... .......... .......... ..........
CW3  .......... .......... .......... .......... .......... ..........
CW4  .......... .......... .......... .......... .......... ..........
CJ2  .......... .......... .......... .......... .......... ..........
CJ3  .......... .......... .......... .......... .......... ..........
CJ5  .......... .......... .......... .......... .......... ..........
SAC2 .......... .......... .......... .......... .......... .T........
SAC3 .......... .......... .......... .......... .......... .T........
SJ1  .......... .......... .......... .......... .......... ..........
SJ2  .......... .......... .......... .......... .......... ..........
SJ3  .......... .......... .......... .......... .......... .T........
K2   .......... .......... .......... .......... .......... ..........
K3   .......... .......... .......... .......... .......... ..........
K4   .......... .......... .......... .......... .......... ..........
SAY2 .......... .......... CC........ ...TT..... ....C..... ..........
SAY3 .......... .......... CC........ ...TT..... ....C..... ..........
SAY4 .......... .......... CC........ ...TT..... ....C..... ..........
SAY5 .......... .......... CC........ ...TT..... ....C..... ..........
SAY6 .......... .......... CC........ ...TT..... ....C..... ..........
AA3  .......... CA........ CC.G...... .....A...A .C.GC..... .T...CCC..
271P .......... CA........ CC.G...... .....A...A .C.GC..... .T...CCC..
SM6  .......... CA........ CC.G...... .....A...A ...GC..... .T...CCC..

                                                                                                                  300
N2   CCACCTAACT CTCAAATTTT AAA--CACAA TAAAAATAAA GCTTCACAGG GTCTTATCGT
CW2  .......... .......... ...--..... .......... .......... ..........
CW3  .......... .......... ...--..... .......... .......... ..........
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```
CW4  .......... .......... ...--..... .......... .......... ..........
CJ2  .......... .......... ...--..... .......... .......... ..........
CJ3  .......... .......... ...--..... .......... .......... ..........
CJ5  .......... .......... ...--..... .......... .......... ..........
SAC2 .......... .......... ...--..... .......... .......... ..........
SAC3 .......... .......... ...--..... .......... .......... ..........
SJ1  .......... .......... ...--..... .......... .......... ..........
SJ2  .......... .......... ...--..... .......... .......... ..........
SJ3  .......... .......... ...--..... .......... .......... ..........
K2   .......... .......... ...--..... .......... .......... ..........
K3   .......... .......... ...--..... .......... .......... ..........
K4   .......... .......... ...--..... .......... .......... ..........
SAY2 T...AC.... A......A.. ...A-..T.. .......... .......... ..........
SAY3 T...AC.... A......A.. ...A-..T.. .......... .......... ..........
SAY4 T...AC.... A......A.. ...A-..T.. .......... .......... ..........
SAY5 T...AC.... A......A.. ...A-..T.. .......... .......... ..........
SAY6 T...AC.... A......A.. ...A-..T.. .......... .......... ..........
AA3  .T..AC..T. A.TG.GCCC. ...AT..A.G C......... .......... ..........
271P .T..AC..T. A.TG.GCCC. ...AT..A.G C......... .......... ..........
SM6  .T..AC.... A.TG.GCCC. ...AT..A.G C......... .......... ..........

                                                                    360
N2   CCCCTTAATT TATTTAAGCC TTTTCACTTA AAAGTAAAAT TCAATT-CTA TATTTGTAGA
CW2  .......... .......... .......... .......... .......-A.. ..........
CW3  .......... .......... .......... .......... .......-... ..........
CW4  .......... .......... .......... .......... .......-... ..........
CJ2  .......... .......... .......... .......... .......-... ..........
CJ3  .......... .......... .......... .......... .......-... ..........
CJ5  .......... .......... .......... .......... .......-... ..........
SAC2 .......... .......... .......... .......... .......-... ..........
SAC3 .......... .......... .......... .......... .......-... ..........
SJ1  .......... .......... .......... .......... .......-... ..........
SJ2  .......... .......... .......... .......... .......-... ..........
SJ3  .......... .......... .......... .......... .......-... ..........
K2   .......... .......... .......... .......... .......-... ..........
K3   .......... .......... .......... .......... .......-... ..........
K4   .......... .......... .......... .......... .......-... ..........
SAY2 ...T...... .......... .......... .......... .....A-... ....C.C...
SAY3 ...T...... .......... .......... .......... .....A-... ....C.C...
SAY4 ...T...... .......... .......... .......... .....A-... ....C.C...
SAY5 ...T...... .......... .......... .......... .....A-... ....C.C...
SAY6 ...T...... .......... .......... .......... .....A-... ....C.C...
AA3  ....C..CAC A......... ........C. .......... .....ATT.. C...C.A...
271P ....C..CAC A......... ........C. .......... .....ATT.. C.C.C.A...
SM6  ....C..CAC A......... ........C. .......... .....ATT.. C.C.C.A...

                                                                    420
N2   CAAATTATAT TTCATCCAAC CATTCATTCC AGACTCCAAT TAAGAGACTA TTGATTATGC
CW2  .......... .......... .......... .......... .......... ..........
CW3  .......... .......... .......... .......... .......... ..........
CW4  .......... .......... .......... .......... .......... ..........
CJ2  .......... .......... .......... .......... .......... ..........
CJ3  .......... .......... .......... .......... .......... ..........
CJ5  .......... .......... .......... .......... .......... ..........
```

```
SAC2 .......... .......... .......... .......... .......... ..........
SAC3 .......... .......... .......... .......... .......... ..........
SJ1  .......... .......... .......... .......... .......... ..........
SJ2  .......... .......... .......... .......... .......... ..........
SJ3  .......... .......... .......... .......... .......... ..........
K2   .......... .......... .......... .......... .......... ..........
K3   .......... .......... .......... .......... .......... ..........
K4   .......... .......... .......... .......... .......... ..........
SAY2 .......... .......G.. .......... .......... .......... ..........
SAY3 .......... .......G.. .......... .......... .......... ..........
SAY4 .......... .......G.. .......... .......... .......... ..........
SAY5 .......... .......G.. .......... .......... .......... ..........
SAY6 .......... .......G.. .......... .......... .......... ..........
AA3  .....C.... .C.....G.. .G.....A.T .......... ...A...... ..........
271P .....C.... .C.....G.. .G.....A.T .......... ...A...... ..........
SM6  .....C.... .C.....G.. .G.....A.T .......... ...A...... ..........

                                                                    480
N2   TACCTTAGCA CGGTCCAAAT ACCGCGGCCC TTTAATAACT CAGTGGGCAG GCCAGACTAA
CW2  .......... .......... .......... .......... .......... ..........
CW3  .......... .......... .......... .......... .......... ..........
CW4  .......... .......... .......... .......... .......... ..........
CJ2  .......... .......... .......... .......... .......... ..........
CJ3  .......... .......... .......... .......... .......... ..........
CJ5  .......... .......... .......... .......... .......... ..........
SAC2 .......... .......... .......... .......... .......... ..........
SAC3 .......... .......... .......... .......... .......... ..........
SJ1  .......... .......... .......... .......... .......... ..........
SJ2  .......... .......... .......... .......... .......... ..........
SJ3  .......... .......... .......... .......... .......... ..........
K2   .......... .......... .......... .......... .......... ..........
K3   .......... .......... .......... .......... .......... ..........
K4   .......... .......... .......... .......... .......... ..........
SAY2 .......... .......... .......... ........A. .......... ..........
SAY3 .......... .......... .......... ........A. .......... ..........
SAY4 .......... .......... .......... ........A. .......... ..........
SAY5 .......... .......... .......... ........A. .......... ..........
SAY6 .......... .......... .......... ........A. .......... ..........
AA3  .......... .......... .......... ........T. .......... ..........
271P .......... .......... .......... ........T. .......... ..........
SM6  .......... .......... .......... ........T. .......... ..........

      484
N2   AATT
CW2  ....
cw3  ....
CW4  ....
CJ2  ....
CJ3  ....
CJ5  ....
SAC2 ....
SAC3 ....
SJ1  ....
SJ2  ....
```

```
SJ3  ....
K2   ....
K3   ....
K4   ....
SAY2 ...G
SAY3 ...G
SAY4 ...G
SAY5 ...G
SAY6 ...G
AA3  ....
271P ....
SM6  ....
```

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